Library of the Medical Department of the University of Leeds and of the Leeds and West Riding Medico-Chirurgical Society.

This Work of Reference is not for circulation and must not be removed from this room.

THE HONORARY LIBRARIAN.

STACK
QS 525
LEE

121 Charing Cross Rd London
FOYLES
THE BOOK SELLERS

30106 004071246

B
279

Digitized by the Internet Archive
in 2015

https://archive.org/details/b21506802

THE

MICROTOMIST'S VADE-MECUM

First Edition	March, 1885.
Second Do.	April, 1890.
Third Do.	September, 1893.
Fourth Do.	September, 1896.
Fifth Do.	June, 1900.
Sixth Do.	June, 1905.
Seventh Do.	August, 1913.

THE

MICROTOMIST'S VADE-MECUM

A HANDBOOK OF THE METHODS OF MICROSCOPIC ANATOMY

BY

ARTHUR BOLLES LEE

SEVENTH EDITION

LONDON
J. & A. CHURCHILL
7, GREAT MARLBOROUGH STREET
1913

PRINTED BY ADLARD AND SON
LONDON AND DORKING

UNIVERSITY OF LEEDS
MEDICAL LIBRARY.

691606

PREFACE.

An important improvement in histological technique, made known since the publication of the last Edition, has been effected by Gilson's new mounting media. For these media—camsal balsam and euparal—afford a ready and safe means of mounting direct from alcohol, without the intervention of essential oils or other clearing agents, which are often contra-indicated; and of conferring on unstained or insufficiently stained elements just the required degree of visibility, thus enabling us to see details which are invisible in the usual mounts.

Some important improvements have also been made in the silver fibril stains of Bielschowsky and Ramón y Cajal, which have now become less capricious methods for the study of neurofibrils, and valuable aids to the study of other objects. I have given these methods at length, abstracting the whole of Ramón's methods from the latest original source. The sections relating to neurofibrils are thus almost entirely re-written, and so are those relating to blood and blood-parasites.

If these are the only novelties of much importance that have offered themselves, yet I have found a large amount of less important matter that it has seemed desirable to include (the Index shows more than 700 new entries). I have been able to find room for this, without increasing the size of the book, partly by striking out some superfluous matter (mostly of merely theoretical interest), and partly by rigorous condensation of the text and not a little typographical compression. To my satisfaction I find that this condensation and compression is not to be regretted, for the text has in many places thereby become easier to understand, and information desired at any time will be more readily found than before.

I have been careful not to carry the typographical compression so far as to make the text trying to the eyes.

Baugy, sur Clarens, Switzerland;
August, 1913.

CONTENTS.

PART I.

PAGE

CHAPTER I.

INTRODUCTORY 1

CHAPTER II.

KILLING 12

CHAPTER III.

FIXING AND HARDENING 19

CHAPTER IV.

FIXING AND HARDENING AGENTS; MINERAL ACIDS AND THEIR SALTS 28

CHAPTER V.

FIXING AND HARDENING AGENTS; CHLORIDES, ORGANIC ACIDS AND OTHERS 45

CHAPTER VI.

DE-ALCOHOLISATION AND CLEARING AGENTS . . . 68

CHAPTER VII.

IMBEDDING METHODS—INTRODUCTION 75

CHAPTER VIII.

IMBEDDING METHODS: PARAFFIN AND OTHER FUSION MASSES . 81
Paraffin, 81; Gelatin, 100.

PAGE

CHAPTER IX.

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS . 102
Collodion or Celloidin, 102; other Cold Masses, 115; Grinding Masses, 115; Freezing, 117.

CHAPTER X.

SERIAL SECTION MOUNTING 119
Methods for Paraffin Sections, 119; Methods for Watery Sections, 125; Methods for Celloidin Sections, 125.

CHAPTER XI.

STAINING 129

CHAPTER XII.

CARMINE AND COCHINEAL STAINS 141
Theory of Carmine Staining, 141; Aqueous Carmines, Acid, 142; Neutral and Alkaline, 146; Alcoholic Carmines and Cochineals, 148.

CHAPTER XIII.

HÆMATEÏN (HÆMATOXYLIN) STAINS 152
Theory of Staining with Hæmatoxylin, 152; Iron-hæmateïn Lakes, 155; Aluminium-hæmateïn Lakes, 159; other Hæmateïn Compounds, 165.

CHAPTER XIV.

NUCLEAR STAINS WITH COAL-TAR DYES 169
Progressive Stains, 170; Regressive Stains, 173.

CHAPTER XV.

PLASMA STAINS WITH COAL-TAR DYES . . . 182

CHAPTER XVI.

METHYLEN BLUE 199

CHAPTER XVII.

METALLIC STAINS (IMPREGNATION METHODS) . . . 212
Silver, 214; Gold, 218; other Metallic Stains, 226.

PAGE

CHAPTER XVIII.

Other Stains and Combinations 228
Other Organic Stains, 228; Carmine Combinations, 229; Hæmateïn Combinations, 231.

CHAPTER XIX.

Examination and Preservation Media . . . 234
Aqueous Liquids, 235; Mercurial Liquids, 239; other Fluids, 239; Glycerin Media, 241; Jellies, 242; High Refractive Liquids, 243; Resinous Media, 244.

CHAPTER XX.

Cements and Varnishes 249

PART II.

SPECIAL METHODS AND EXAMPLES.

CHAPTER XXI.

Injections: Gelatin Masses (warm) 257
Carmine, 259; Blue, 261; other Colours, 262.

CHAPTER XXII.

Injections: other Masses (cold) 264

CHAPTER XXIII.

Maceration, Digestion, and Corrosion . . . 270
Maceration, 270; Digestion, 276; Corrosion, 277.

CHAPTER XXIV.

Decalcification, Desilicification, and Bleaching . 279
Decalcification, 279; Desilicification, 283; Bleaching, 284.

CHAPTER XXV.

Embryological Methods 287
Mammalia, 291; Aves, 295; Reptilia, 299; Amphibia, 300; Pisces, 303; Tunicata, 306; Bryozoa, 306; Mollusca, 307; Arthropoda, 309; Vermes, 314.

CHAPTER XXVI.

Cytological Methods 317

PAGE

CHAPTER XXVII.

TEGUMENTARY ORGANS 331

CHAPTER XXVIII.

MUSCLE AND TENDON (NERVE-ENDINGS) 337
Striated Muscle, 337; Electric Organs, 339; Tendon, 340; Smooth Muscle, 341.

CHAPTER XXIX.

Connective Tissue, 344; Elastic, 346; Plasma Cells, 349; Fat, 351; Bone and Cartilage, 353.

CHAPTER XXX.

BLOOD AND GLANDS 359
Blood, 359; Glands, 368.

CHAPTER XXXI.

NERVOUS SYSTEM: GENERAL METHODS 373

CHAPTER XXXII.

NERVOUS SYSTEM: CYTOLOGICAL METHODS . . . 386
Cells, 386; Cells and Fibres, 389; Medullary Sheath, 404.

CHAPTER XXXIII.

MYELIN STAINS (WEIGERT AND OTHERS) 407

CHAPTER XXXIV.

AXIS-CYLINDER AND DENDRITE STAINS (GOLGI AND OTHERS) . 419

CHAPTER XXXV.

NEUROGLIA AND SENSE ORGANS 438
Neuroglia, 438; Retina, 442; Inner Ear, 445.

CHAPTER XXXVI.

METHODS FOR INVERTEBRATES 448
Tunicata, 448; Molluscoida, 449; Mollusca, 449; Arthropoda, 454; Vermes, 458; Echinodermata, 467; Cœlenterata, 470; Porifera, 474; Protozoa, 475.

APPENDIX 484

THE MICROTOMIST'S VADE-MECUM.

CHAPTER I.

INTRODUCTORY.

1. **The General Method.**—The methods of modern microscopic anatomy may be roughly classed as General and Special. There is a General or Normal method which consists in carefully *fixing* the structures to be examined, *staining* them with a *nuclear* stain, *dehydrating* with alcohol, and mounting *series of sections* of the structures in *balsam*. It is by this method that the work is blocked out and very often finished. Special points are then studied, if necessary, by Special Methods, such as examination of the living tissue elements, *in situ* or in "indifferent" media; fixation with special fixing agents; staining with special stains; dissociation by teasing or maceration; injection; impregnation; and the like.

There is a further distinction which may be made, and which may help to simplify matters. The processes of the preparation of tissues may be divided into two stages, *Preliminary Preparation* and *Ulterior Preparation*. Now the processes of preliminary preparation are essentially identical in all the methods, essential divergences being only found in the details of ulterior preparation. By preliminary preparation is meant that group of processes whose object it is to get the tissues into a fit state for passing unharmed through all the ulterior processes to which it may be desired to submit them. It comprehends the operations of (1) killing; (2) fixing; (3) the washing and other manipulations necessary for removing the fixing agent from the tissues, and substituting for it the preservative liquid or other reagents which it is desired to employ. Ulterior preparation comprehends the processes sketched out in §§ 3 *et seq.*

2. Preliminary Preparation.—The first thing to be done with any structure is to *fix* its histological elements. (This statement applies equally to all classes of objects, whether it be desired to cut them into sections or to treat them in any other special way.) Two things are implied by the word "fixing": first, the rapid *killing* of the element, so that it may not have time to change the form it had during life, but is fixed in death in the attitude it normally had during life; and second, the *hardening* of it to such a degree as may enable it to resist without further change of form the action of the reagents with which it may subsequently be treated. Without good fixation it is impossible to get *good stains* or good sections, or preparations good in any way.

The structure having been duly fixed by one of the processes described in the chapter on Fixing Agents, is, except in special cases, *washed* in order to remove from the tissues as far as possible all traces of the fixing reagent.

The kind of liquid with which washing out is done is not a matter of indifference. If corrosive sublimate (for instance), or osmic acid, or a solution into which chromic acid or a chromate enters, have been used for fixing, the washing may be done with water. But if certain other agents, such as picric acid, have been used, the washing should be done with alcohol. The reason of this difference is that the first-named reagents (and, indeed, all the compounds of the heavy metals used for fixing) enter into a state of chemical combination with the elements of tissues, rendering them insoluble in water; so that the hardening induced by these agents is not removed by subsequent treatment with water. Picric acid, on the other hand, produces only a very slight hardening of the tissues, so that the tissue elements are left in a state in which they are obnoxious to all the hurtful effects of water. Alcohol should therefore be taken to remove the picric acid and to effect the necessary hardening at the same time.

These operations having been duly performed, two roads become open. The object may be further prepared by what may be termed the *wet* method, in which all subsequent operations are performed by means of aqueous media. Or it may be further prepared by the *dehydration* method, which consists in treatment with successive alcohols of gradually increasing strength, final *dehydration* with absolute alcohol, imbibition with an essential oil or other so called *clearing agent* which serves to remove the alcohol, and lastly either mounting at once in balsam or other resinous medium or imbedding in paraffin for the purpose of making sections. The dehydration method is the course which is generally preferred, chiefly because of its great superiority as regards the preservation of tissues. For the presence of water is the most important factor in the conditions that bring about the decomposition of organic matter, and its complete removal is the chief condition of permanent preservation.

3. **Dehydration.**—The further course of preparation by the dehydration method is as follows:—At the same time that the superfluous fixing agent is being removed from the tissues, or as soon as that is done, the *water of the tissues must be removed.* This is necessary for two reasons: first, in the interest of preservation, as above explained; and secondly, because all water must be removed in order to allow the tissues to be impregnated with the imbedding material necessary for section-cutting, or with the balsam with which they are to be finally preserved. This *dehydration* is performed as follows:—The objects are brought into weak alcohol, and are then passed through successive alcohols of gradually increased strength, remaining in each the time necessary for complete saturation, and the last bath consisting of absolute or at least very strong alcohol.

In dealing with delicate objects, it may be necessary to take special precautions in order to avoid injury to them through the violent diffusion-currents that are set up in the passage from water to alcohol, or from one bath of alcohol to another of considerably different density. Some kind of diffusion-apparatus may conveniently be used in these cases. The objects may be placed with some of their liquid in a tube corked at one end and closed at the other by a diaphragm of muslin or chamois skin or other suitable membrane, the tube being then immersed in a vessel containing the grade of alcohol that it is desired to add to the liquid in the tube, and the whole allowed to remain until by diffusion through the diaphragm the two liquids have become of equal density. Or, COBB'S differentiator (*Proc. Linn. Soc.*, N.S.W., v, 1890, p. 157; *Journ. Roy. Mic. Soc.*, 1890, p. 821) may be employed. Or, the apparatus of HASWELL (*Proc. Linn. Soc.*, N.S.W., vi, 1891, p. 433; *Journ. Roy. Mic. Soc.*, 1892, p. 696). Or that of CHEATLE, described in *Journ. Pathol. and Bacteriol.*, i, 1892, p. 253, or *Journ. Roy. Mic. Soc.*, 1892, p. 892. See also SCHULTZE (*Zeit. wiss. Mik.*, ii, 1885, p. 537); and SUSUKI, *ibid.*, 1909, p. 211; KOLSTER (*ibid.*, xvii, 1900, p. 294).

The "Siebdosen," or sieve-dishes of STEINACH, ZIMMERMANN, and SUCHANNEK (vide *Zeit. wiss. Mik.*, iv, 1887, p. 433, and vii, 1890, p. 158), are useful for many purposes. They are sent out in a very neat form by Grübler and Co. See also TISCHATKIN, *ibid.*, xxiii, p. 45. FAIRCHILD'S perforated porcelain cylinders for washing (*ibid.*, xii, 1896, p. 301) seem to be a very neat idea. See also the similar device of SCHAFFER (*ibid.*, xvi, 1900, p. 422; *Journ. Roy. Mic. Soc.*, 1900, p. 394). For EWALD'S section-washing apparatus, see *Zeit. Biol.*, xxxiv, 1897, p. 264.

That of SCHOEBEL (*ibid.*, xx, 1903, p. 168) is simple and efficient; as also that of KRIEGBAUM (*ibid.*, xxvii, 1910, p. 504).

A *capillary siphon* for the aspiration of liquids in the fixing, staining, and washing of suspended blood-corpuscles, sperm-cells, protozoa, and the like, is described by EWALD, *ibid.*, p. 253.

It is sometimes stated that it is necessary that the last alcohol-bath should consist of absolute alcohol. This, however, is incorrect, a strength of 95 per cent. being sufficient in most cases. For the small amount of water that remains in the tissues after treatment with these grades of alcohol is efficiently removed in the bath of clearing agent if a good clearing agent be employed. Oil of cedar will remove the remaining water from tissues saturated with 95 per cent. alcohol; oil of bergamot will "clear" from 90 per cent. alcohol, and anilin oil will clear from 70 per cent. alcohol.

I am not aware of any substance that can entirely take the place of alcohol for dehydration and preservation. Acetone and methylal have been substituted for alcohol in the dehydration of methylen-blue preparations (PARKER, *Zool. Anz.*, 403, 1892, p. 376), and anilin oil can be made to dehydrate watery sections if they be first mopped up with blotting-paper; but a really efficient substitute for alcohol in general work remains yet to be discovered.

4. Preservation.—Considered as a mere dehydrating agent, *alcohol* fulfils its functions fairly well. But considered as a histological *preservative* agent, it is far less satisfactory. If tissues be left in alcohol for only a few days before further preparation, injurious effects will perhaps not be very disagreeably evident. But it is otherwise if they are put away in it for many weeks or months before the final preparation is carried out. The dehydrating action of the alcohol being continuously prolonged, the minute structure of tissues is sometimes considerably altered by it; they become over-hard and shrink, and become brittle, and their capacity for taking stains well becomes seriously diminished. KULTSCHITZKY (*Zeit. wiss. Mik.*, iv, 1887, p. 349) has proposed to remedy this by putting up objects after fixation and washing out with alcohol in ether, xylol, or toluol. FLEMMING (*Arch. mik. Anat.*, xxxvii, 1891, p. 685) advises putting up objects after fixation in a mixture of alcohol, glycerin, and water, in about equal parts, pointing out that objects thus preserved may be at any moment either prepared for sectioning by treatment with pure alcohol or softened for dissection or teasing by a little soaking in water, and that they do not become so hard and brittle as alcohol specimens, and retain their staining power much better. After extensive experience of this plan I can recommend it, and would only further suggest that the action of the liquid seems to me to be in many cases much improved by addition of a little acetic acid (say 0·5 to 0·75 per cent.).

For material that is intended *only for section-cutting*, I find that by far the best plan is to clear (next §) and imbed at once in paraffin. This affords, as far as I can see, an absolutely perfect preservation. Cedar-wood oil is, I find, nearly, if not quite, as good as paraffin, so far as the preservation of the tissues is concerned, but of course it is not so handy for storage.

5. **Removal of Alcohol; Clearing.**—The water having been sufficiently removed, as described in § 3, the alcohol is in its turn removed from the tissues, and its place taken by some anhydrous substance, generally an essential oil, which is miscible with the material used for imbedding or mounting. This operation is generally known as *Clearing.* It is very important that the passage from the last alcohol to the clearing agent be made *gradual.* This is effected by placing the clearing medium *under* the alcohol. A sufficient quantity of alcohol is placed in a tube (a watch-glass will do, but tubes are generally better), and then with a pipette a sufficient quantity of clearing medium is introduced *at the bottom of the alcohol.* Or you may first put the clearing medium into the tube, and then carefully pour the alcohol on to the top of it. The two fluids mingle but slowly. The objects

to be cleared, being now quietly put into the supernatant alcohol, float at the surface of separation of the two fluids, the exchange of fluids takes place gradually, and the objects slowly sink down into the lower layer. When they have sunk to the bottom, the alcohol may be drawn off with a pipette, and after some further lapse of time the objects will be found to be completely penetrated by the clearing medium.

This method of making the passage from one fluid to another applies to all cases in which objects have to be transferred from a lighter to a denser fluid—for instance, from alcohol, or from water, to glycerin.

This is a convenient stage for carrying out *minute dissections*, if any such have to be done, a drop of clearing agent being a most helpful medium for carrying out such dissections (see § 9).

At this point the course of treatment follows one of two different roads, according as the object is to be mounted direct in balsam (§ 8), or is first to be sectioned (§ 6).

6. Imbedding, and Treatment of Sections.—The objects are now *imbedded*. They are removed from the clearing medium, and soaked until thoroughly saturated in the imbedding medium. This is, for small objects, generally paraffin, liquefied by heat, and for large objects either paraffin or a solution of collodion or "celloidin" (in this latter case the clearing may be omitted and the tissues be imbedded direct from the alcohol). The imbedding medium containing the object is then made to solidify, and sections are made with a microtome through the imbedding mass and the included objects. The sections are then mounted on a slide by one of the methods described in the chapter on Serial Section Methods, the imbedding material is removed from them (in the case of paraffin), they are stained *in situ* on the slide, dehydrated with alcohol, cleared, and mounted in balsam or damar. Or they may be stained, washed, dehydrated, and cleared in watch-glasses, and afterwards mounted as desired—the imbedding medium being first removed if desirable.

Or, the material may be stained in bulk, before cutting the sections. In this case the object, after having been fixed and washed out, is taken from the water, or while still on its way through the lower alcohols (it should not be allowed to proceed to the higher grades of alcohol before staining, if that can be avoided), and passed through a bath of stain, then dehydrated with successive alcohols, passed through a clearing medium into paraffin, cut, and treated as above described, the sections in this case being mounted direct from the chloroform, xylol, or other solvent with which the paraffin is removed. If aqueous staining media be applied (and this is sometimes desirable), the structures should either be stained *in toto* immediately after fixing and washing out, or sections may be stained on the slide, the objects, if delicate, being passed through successive baths of alcohol of gradually decreasing strength before being put into the aqueous stain.

In my opinion it is generally advisable not to stain in bulk material that is intended to be sectioned; by staining it as sections the staining can be much better controlled, and many excellent stains can in this way be employed that are not available for staining in bulk; and of course sections can be stained much more rapidly than material in bulk.

Balsam mounts of which the stain has faded, or which it may be desired to submit to some other staining process, or mount in some other medium, may often with great advantage be *re-strained* and *re-mounted.* All that is necessary is to put the slide into a tube of xylol or benzol till the cover falls off (about two days), wash well for some hours in clean xylol, and pass through alcohol into the new stain. Since this was pointed out to me by Dr. Henneguy I have unmounted and re-stained a large number of old preparations, and have succeeded in every case with series of sections mounted on Mayer's albumen, or by the water method. For shellac-mounted series, *see* E. MEYER, *Biol. Centralb.*, x, 1890, p. 509, or *last edition.*

The most convenient *vessels*, I find, in which to perform the various operations of staining, differentiating, dehydrating, clearing, etc., *on the slide*, are flat-bottomed corked glass tubes. I have mine made 10 centimetres high and 27 millimetres internal diameter. Each of these will then take two slides, English size, placed back to back.

7. Résumé of the General Method.—To sum up, you may either fix, wash out, stain, wash, dehydrate, clear, imbed, cut sections, clear and mount them in balsam; or fix, wash, dehydrate, clear, imbed, cut, stain, wash, dehydrate, clear, and mount—according to choice.

8. Preparation of Entire Objects, or of Material that is not to be sectioned.—The treatment of objects which can be studied without being cut into sections is identical with that above described, with the omission of those passages that relate to imbedding processes. Its normal course may be described as fixation, washing out, staining, treatment with successive alcohols of gradually increasing strength, final dehydration with absolute alcohol, clearing, and mounting in balsam.

In the *preparation of entire objects* or structures that are intact and *covered by an integument not easily permeable* by liquids, special care must be taken to avoid swelling from endosmosis on the passage of the objects from any of the liquids employed to a liquid of less density, or shrinkage from exosmosis on the passage to a liquid of greater density. This applies most specially to the passage from the last alcohol into the clearing medium. A slit should be made in the integument, if possible, so that the two fluids may mingle without hindrance. And in all cases the passage is made gradual by placing the clearing medium under the alcohol, as described (§ 5). Fluids of high diffusibility should be employed as far as possible in all the processes. Fixing agents of great penetrating power (such as picric acid or alcoholic sublimate solution) should be employed where the objects present a not easily permeable integument. Washing out is done with successive alcohols, water being used only in the case of fixation by osmic acid, or the chromic mixtures or other fixing solutions that render washing by water imperative. Staining is done by preference with alcoholic staining media. The stains most to be recommended are Grenacher's borax-carmine, or one of Mayer's alcoholic carminic acid or hæmatein stains. Aqueous stains are more rarely indicated, though there are many cases in which they are admissible, and some in which they are preferable.

9. Minute Dissections.—These are best done, if necessary, in a drop of clearing agent. I recommend cedar-wood oil for this purpose, as it gives to the tissues a consistency very favourable for dissection, whilst its viscosity serves to lend support to delicate structures. Clove oil has a tendency to make tissues that have lain in it for some time very brittle.

The brittleness is, however, sometimes very helpful in minute dissections. Another property of clove oil is that it does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops, and this also makes it frequently a very convenient medium for making minute dissections in.

If it be desired to dissect in a watery fluid, such as glycerin, it may be well to prepare the slide by spreading on it a thin layer of MAYER's albumen, and on this place a small drop of glycerin, or other dissecting medium. As soon as the dissection has been accomplished, a cover may be let fall, horizontally, on to the preparation to keep the parts in place, and a weight placed on it. Then the mount may be filled up with glycerin, or other mounting medium, run in under the cover, and closed, if desired, or instead of the albumen a solution of gelatin may be taken, and hardened in formol with the objects on it. For a balsam mount, after clove or cedar oil, SCHÄLLIBAUM's collodion may be taken, and the organs fixed *in situ* on this by adding xylol.

10. Instruments.—For all that concerns the mechanism and manipulation of *the Microscope,* see vol. i of CARPENTER's *The Microscope,* eighth edition, 1891; paying particular attention to all that is said concerning the *English* and the *Continental Models,* pp. 254 to 261, the *Substage,* pp. 184 to 189, *Condensers,* pp. 289 to 316, and *Tube Length,* pp. 158 to 159.

For information concerning the principles of construction and manipulation of *the Microtome,* see also CARPENTER's *The Microscope.* Microtomes are instruments for the accurate production of thin slices of tissues. They are used both for cutting tissues that have acquired a certain favourable consistency through having been *imbedded* in *paraffin,* and also for cutting tissues that have been imbedded in softer masses, such as *collodion,* and tissues that have *not been imbedded* at all. Not all microtomes are equally well adapted for all these three classes of work. The microtome of the zoologist should at all events be one that is well adapted for cutting imbedded material.

Now there are two methods of imbedding in general use —the paraffin method and the celloidin method. In the paraffin method the object is cut *dry,* frequently with the knife set *square* to the line of section. In the celloidin method, as in the cutting of unimbedded tissues, it is generally cut *wet*, and always with the knife set *slanting*. Some microtomes that are well adapted for the paraffin method are ill adapted for the celloidin method or the cutting of unimbedded material, and *vice versâ*. It may be well to possess the two sorts of instrument; but if only one can be afforded it should be such as will give good work in either way.

Microtomes fall further into two classes according as the knife and the surface of section of the object are (A) in a horizontal plane, or (B) in a vertical plane. The former offer greater facility for the orientation of the plane of section, which is an important point for the zoologist and embryologist. Amongst these may be mentioned (*a*) The "Sliding" Microtomes, in which the knife is carried on a sledge and moved against the object (those of THOMA, SCHANZE, REICHERT, and others). The THOMA, of medium size, as made by R. Jung, Hebelstrasse, Heidelberg (No. 56 of his catalogue for 1911, which may be obtained from Mr. C. Baker, 244, High Holborn, London), is very suitable for the zoologist. It works equally well with either paraffin or celloidin, and can be adapted as a freezing microtome. But this (as is the case with the others mentioned) will not always furnish work of the highest accuracy; for the knife being only clamped at one end is liable to spring, and to give sections of unequal thickness. This defect is remedied in (*b*), a type of sliding microtomes in which the knife is clamped at both ends and is a fixture, the object being carried on a sledge and moved against it (CAMBRIDGE SCIENTIFIC INSTRUMENT COMPANY'S large microtome, the MINOT precision microtome, LEITZ'S, DE GROOT'S, JUNG'S "Tetrander." This last seems to be near perfection; see the description by MAYER in *Zeit. wiss. Mik.*, xxvii, 1910, p. 52; but is more cumbrous than is desirable for ordinary work.

Class A also includes some instruments in which the knife is carried on a horizontal arm and swung against the object by a rotary movement (JUNG, ROY, FROMME, REICHERT, THATE, and others). I know nothing of these personally, but doubt their constant accuracy.

Class B contains some very fine instruments, admirably adapted for the production of continuous ribbons of sections by the paraffin method, but not so well adapted for celloidin or other work in the wet way, or for soft objects. Amongst these are the Rocking Microtome, made by THE SCIENTIFIC INSTRUMENT Co., Cambridge, or by SWIFT & SON, or by JUNG, or by VAN DER STAD, Amsterdam; the MINOT, made by BAUSCH & LOMB, or by BECKER (Göttingen), or by Zimmermann (21, Emilienstrasse, Leipzig); the REINHOLD-GILTAY, made by J. W. GILTAY, Delft.

For descriptions of the multitudinous models on the market see the reports in the *Zeit. wiss. Mik.* and *Journ. Roy. Micr. Soc.*, and the price lists of the instrument makers.

11. Staining Reagents and Chemicals.—You are not likely to succeed in staining, especially in staining with coal-tar colours, unless you see to it that you are working with chemicals of the proper quality. You *cannot* ensure this by going to a generally trustworthy house for chemical products —at all events, not in the case of coal-tar colours. It is not sufficient that these should be what they are commercially described to be; they may be pure, and yet not give good stains. They must (in the case of coal-tar colours, at all events) be the identical products used in their work by the authors who have described and recommended them (see the note on the numerous safranins in the market, *s. v. Safranin*). I therefore advise the reader to get his reagents—at all events his dyes—from the well-known chemists GRÜBLER & HOLLBORN, who have specialised in the matter. They may be ordered from the price list, or by quoting the numbers of the formulæ in this work. The address is: G. GRÜBLER & HOLLBORN, Chemiker, Leipzig, Germany. Their preparations can be obtained in London from Mr. CHARLES BAKER, 244, High Holborn, W.C., or H. F. ANGUS & Co., 83, Wigmore Street, Cavendish Square. (Both of these firms also supply the microtomes and accessory apparatus of the best makers, bacteriological apparatus, etc.) Grübler & Hollborn's preparations can be obtained in the States from EIMER & AMEND, 205–211, Third Avenue, New York; PAUL WEISS, Optician, 1620, Arapahoe Street, Denver, Colorado; and EDWARD P. DOLBEY & Co., 3613, Woodland Avenue, Philadelphia.

CHAPTER II.

KILLING.

12. In the majority of cases, the first step in the preparation of an organ or organism consists in exposing it as rapidly and as completely as possible to the action of one of the **Fixing Agents** that are discussed in the next chapter. The organ or organism is thus taken in the normal living state; the fixing agent serves to bring about at the same time, and with sufficient rapidity, both the death of the organism and that of its histological elements.

But this method is by no means applicable to all cases. There are many animals, especially such as are of a soft consistence, and deprived of any rigid skeleton, but possessing a considerable faculty of contractility, which if thus treated contract violently, and die in a state of contraction that renders them unfit for study. In these cases special methods of killing must be resorted to. Speaking generally, there are two ways of dealing with these difficult cases. You may kill the animal so suddenly that it has not time to contract: or you may paralyse it by narcotics before killing it.

Sudden Killing.

13. Heat.—The application of *Heat* affords a means of killing suddenly. By it the tissues are more or less fixed at the same time that somatic death is brought about.

The difficulty consists in hitting off the right temperature, which is of course different for different objects. I think that 80° to 90° C. will generally be amply sufficient, and that very frequently it will not be necessary to go beyond 60° C. An exposure to heat for a few seconds will generally suffice.

Small objects (Protozoa, Hydroids, Bryozoa) may be brought into a drop of water in a watch-glass or on a slide, and heated over the flame of a spirit-lamp. For large objects, the water or other liquid employed as the vehicle of the heat may be heated beforehand and the animals thrown into it.

As soon as it is supposed that the protoplasm of the tissues is coagulated throughout, the animals should be brought into alcohol (30 to 70 per cent. alcohol) (if water be employed as the heating agent).

An excellent plan for preparing many marine animals is to kill them in *hot fresh-water*. Some of the larger Nemertians are better preserved by this method than by any other with which I am acquainted.

14. **Slowly Contracting Animals.**—Animals that contract but slowly, such as *Alcyonium* and *Veretillum*, and some Tunicates, such as *Pyrosoma*, are very well killed by throwing them into some very quickly acting fixing liquid, either used hot or cold. *Glacial* or very strong *acetic acid* (VAN BENEDEN'S method) is an excellent reagent for this purpose; it may be used, for example, with some Medusæ. After an immersion of a few seconds or a few minutes, according to the size of the animals, they should be brought into alcohol of at least 50 per cent. strength. *Lemon juice* employed in this way has given me very good results with small Annelids and Hirudinea. *Corrosive sublimate* is another excellent reagent for this purpose.

Narcotisation.

15. **Narcotisation** is performed by adding some anæsthetic substance very gradually, in very small doses, to the water containing the animals, and waiting patiently for it to take effect slowly.

The Tobacco-smoke Method for Actiniæ, of LO BIANCO (*Jena Zeit. Naturw.*, Bd. xiii, 1879, p. 467; *Mitth. Zool. Stat. Neapel*, Bd. ix, 1890, p. 499), is:—A dish containing the animals in water is covered with a bell-glass, under which passes a curved glass or rubber tube, which dips into the water. Tobacco smoke is blown into the water for some time through the tube, and the animals are then left for some hours overnight. As soon as it is observed that the contraction of a tentacle does not begin until a considerable time after it has been irritated by a needle, a quantity of some fixing liquid sufficient to kill the animals before they have time to contract is added to the water.

16. **Nicotin in solution** may be used (ANDRES, *Atti R. Accad. dei Lincei*, v, 1880, p. 9). Andres employs a solution of

1 gramme of nicotin in a litre of sea water. The animal is placed in a jar containing half a litre of sea water, and the solution of nicotin is gradually conducted into it by means of a thread, acting as a syphon, of such a thickness as to be capable of carrying over the whole of the solution of nicotin in twenty-four hours. See also *Mitth. Zool. Stat. Neapel*, Bd. ii, 1880, p. 123.

17. **Chloroform** may be employed either in the liquid state or in the state of vapour. The animals being extended, a watch-glass containing chloroform may be floated on the surface of the water in which they are contained, and the whole covered with a bell-glass. As soon as they have become insensible they are killed by means of hot sublimate or chromic acid solution plentifully poured on to them. (Korotneff, *Mitth. Zool. Stat. Neapel*, v, 1884, p. 233).

Liquid chloroform is employed by squirting it in small quantities on to the surface of the water containing the animals. A syringe or pipette having a very small orifice, so as to thoroughly pulverise the chloroform, should be employed. Small quantities only should be projected at a time, and the dose should be repeated every five minutes until the animals are anæsthetised.

I have seen large Medusæ very completely anæsthetised in extension in an hour or two by this method. Andres finds that it does not succeed with Actiniæ, as with them maceration of the tissues supervenes before anæsthesia is established.

Preyer (*Mitt. Zool. Stat. Neapel*, Bd. vii, 1886, p. 27) recommends chloroform water for star-fishes.

Waddington employs a mixture of equal parts of 1 per cent. sol. of cocaine (or eucain) and saturated sol. of chloroform in water (sea or fresh), according to the habitat.

18. **Ether and Alcohol** may be administered in the same way. Andres has obtained good results with Actiniæ by the use of a mixture (invented by Salvatore lo Bianco) containing 20 parts of glycerine, 40 parts of 70 per cent. alcohol, and 40 parts of sea water. This mixture should be carefully poured on to the surface of the water containing the animals, and allowed to diffuse quietly through it. Several hours are sometimes necessary for this.

Eisig (*Fauna u. Flora Golf. Neapel,* 16, 1887, p. 239) benumbs Capitellidæ by putting them into a mixture of one part of 70 per cent. alcohol with 9 parts of sea water.

Oestergren (*Zeit. wiss. Mik.,* xix, 1903, p. 300) makes a saturated (7 to 8 per cent.) solution of ether in sea or soft water, and uses it either concentrated or diluted to about 1 per cent., and finds it to succeed with all classes of aquatic animals.

Cori (*Zeit. wiss. Mik.,* vi, 1890, p. 438) recommends a mixture composed of 10 c.c. *methyl-alcohol* (of 96 per cent. strength), 90 c.c. water (fresh or sea water), and 0·6 grm. of sodium chloride (to be added only when fresh water is taken, the addition of the salt having for its object to prevent maceration). It may be well to add to this mixture a very few drops of chloroform (for *Cristatella ; Zeit. wiss. Zool.,* lv, 1893, p. 626).

19. Chloreton (Aceton Chloroform) is recommended for invertebrates and larvæ of *Rana* by Randolph (*Zool. Anz.,* xxiii, 1900, p. 436). Krecker (*Zeit. wiss. Zool.,* xcv, 1910, p. 383) takes solutions of $\frac{1}{3}$ to 1 per cent. for Oligochæta. Sulima (*Zeit. Biol. Techn.,* Strasburg, i, 1909, p. 379) takes a mixture of 99 parts of sea water and 1 of 10 per cent. sol. of chloreton in absolute alcohol, for *Scyllium* and *Anguilla.*

20. Hydrate of Chloral.—Foettinger (*Arch. de Biol.,* vi, 1885, p. 115) operates by dropping crystals of chloral into the water containing the animals. For *Alcyonella* he takes 25 to 80 centigrammes of chloral for each hundred grammes of water. It takes about three-quarters of an hour to render a colony sufficiently insensible. He has obtained satisfactory results with marine and fresh-water Bryozoa, with Annelida, Mollusca, Nemertians, Actiniæ, and with *Asteracanthion.* He did not succeed with Hydroids.

Lo Bianco (*Mitth. Zool. Stat. Neapel,* Bd. ix, 1890, p. 442) employs for various marine animals freshly prepared solutions of chloral in sea water, of from one tenth to one fifth per cent. strength.

I have never had the slightest success with Nemertians.

Verworn (*Zeit. wiss. Zool.,* xlvi, 1887, p. 99) puts *Cristatella* for a few minutes into 10 per cent. solution of chloral, in which the animals sooner or later become extended.

Kükenthal (*Jena Zeit. Naturw.*, Bd. xx, 1887, p. 511) has obtained good results with some Annelids by means of a solution of one part of chloral in 1000 parts of sea water.

The chloral method gives rise to maceration with some subjects, as I can testify, and has been said to distort nuclear figures.

21. Cocaine (Richards, *Zool. Anz.*, 196, 1885, p. 332).—Richards puts a colony of Bryozoa into a watch-glass with 5 c.c. of water, and adds gradually 1 per cent. solution of hydrochlorate of cocaine in water. After five minutes the animals are somewhat numbed, and half a cubic centimetre of the solution is added; and ten minutes later the animals should be found to be dead in a state of extension.

This method is stated to succeed with Bryozoa, *Hydra*, and certain worms. It is the best method for Rotifers (Rousselet). It has also been recommended for *Aplysia*.

It has been pointed out (by Cori, in the paper quoted § 18) that, unfortunately, when fixing agents, such as sublimate solution, are added to the animals, the cocaine is thrown down on them as a white precipitate. This precipitate, however, may be redissolved afterwards in alcohol (Eisig).

Cocaine solutions cannot be depended on to keep for more than a few days.

22. Eucain. Harris (*Journ. Roy. Mic. Soc.*, 1900, p. 404) recommends a 1 per cent. solution of eucain hydrochloride, as giving far better results, with Vorticellidæ, Rotatoria, and Vermes. Rousselet (*ibid.*) reports favourably as to its action on Floscularïæ. It is stated to be perfectly stable in aqueous media. It dissolves in sea water to about 0·5 per cent.

23. Hydroxylamin.—Hofer (*Zeit. wiss. Mik.*, vii, 1890, p. 318). Either the sulphate or, preferably, the hydrochlorate may be used. This should be dissolved in water (spring or sea water, according to the habitat) and exactly neutralised by addition of carbonate of soda. The organisms are placed in a solution diluted to about 0·1 per cent., for thirty minutes or less (as for Infusoria), to 0·25 per cent., for from fifteen minutes to one hour (*Hydra*), 1 per cent., one half to two hours (*Hirudo*), or as much as ten to twenty hours (*Helix* and *Anodonta*).

Hydroxylamin is a powerful reducing agent, and should therefore be well washed out before treating with easily reducible fixing agents.

24. Chloride or Sulphate of Magnesium.—Tullberg (*Arch. Zool. Expér. et Gen.*, x, 1892, p. 11). For Actiniæ, a 33 per cent. solution of the chloride should be very slowly added to the water containing the expanded animal, until the vessel contains 1 per cent. of the salt (thus for one litre of sea water 33 c.c. of the solution must be added). The addition must be completed within half an hour, and thirty minutes later the animal may be fixed.

For terrestrial and fresh-water Invertebrates rather stronger solutions should be used.

Redenbaugh (*Amer. Natural*, xxix, 1895, p. 399) takes the sulphate, either added in crystals to the sea water containing the animals until a saturated solution is obtained, or in the shape of a saturated solution into which they are thrown (Annelids.)

See also Mayer, *Biol. Bull. Wood's Hole*, xvii, 1909, p. 341 (puts direct into sol. of $\frac{3}{8}$ strength).

25. Poisoning by small doses of some fixing agent is sometimes good. Lo Bianco kills *Ascidia* and *Rhopalæa* in an extended state (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 471) by pouring a little 1 per cent. chromic acid on to the surface of the water containing them, and allowing it to slowly diffuse into it. About twelve to twenty-four hours is necessary. He kills *Ciona* in a similar way with a mixture of one part of 1 per cent. chromic acid and nine parts of 49 per cent. acetic acid.

Osmic acid, or Kleinenberg's solution, is sometimes employed in the same way.

I have seen Medusæ killed in a satisfactory manner by means of crystals of corrosive sublimate added to the water containing them.

Morphia, Curare, Strychnin, Prussic Acid, and other paralysing drugs, have also been employed.

26. Asphyxiation may be sometimes successfully practised. Terrestrial Gastropods may be killed for dissection by putting them into a jar quite full of water that has been deprived of its air by boiling, and hermetically closed. After from twelve to twenty-four hours they are generally found dead and extended. The effect is obtained somewhat quicker if a little *tobacco* be added to the water.

Good results are sometimes obtained with aquatic animals by simply leaving them to exhaust the oxygen of the water in which they are contained. I have sometimes succeeded with

Holothuriæ and other Echinoderms in this way. Ward (see *Amer. Nat.*, xxv, 1891, p. 398) has succeeded with Hydroids, Actiniæ, and similar forms, and Uexküll (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 463) with Echinids.

Marine animals are sometimes successfully killed by simply putting them into *spring water.*

Warm Water will sometimes serve to immobilise and even kill both marine and fresh-water organisms.

Carbonic Acid Gas has been recommended (by Fol, *Zool. Anz.*, 128, 1885, p. 698). The water containing the animals should be saturated with the gas. The method is stated to succeed with most Cœlenterata and Echinodermata, but not with Molluscs or Fishes. I have had most excellent results with small Annelids and Hirudinea. It is not necessary to employ a generator for obtaining the gas. It suffices to take an ordinary "soda-water" syphon, and squirt its contents into the water containing the animals.

Narcotisation is very rapidly obtained with very small animals, but much more slowly with larger ones. For instance, *Stylaria proboscidea,* I find, is paralysed in a few *seconds*; a small *Nephelis* of 15 or 20 millimetres in length, will require about five *minutes*; and a large Nephelis, of from 10 to 15 centimetres, will require as many *hours.*

Uexküll (*Mitth. Zool. Stat. Neapel,* xii, 1896, p. 463) has paralysed Echinids very rapidly with carbonic acid, likewise a small Teleostean fish; whilst *Scyllium* and Crustaceans were affected much more slowly, and mussels not at all.

27. Peroxide of Hydrogen.—Volk (*Zool. Anz.*, xix, 1896, p. 294) kills Rotatoria by means of one or two drops of a 3 per cent. solution added to 1 c.c. of the water containing them.

CHAPTER III.

FIXING AND HARDENING.

28. The Functions of Fixing Agents.—The meaning of the term "fixing" has been explained above (§ 2). Here is an example showing the necessity of fixation. If a portion of living retina be placed in aqueous humour, serum, or other so-called "indifferent" medium, or in any of the media used for permanent preservation, it will be found that the rods and cones will not preserve the appearance they have during life for more than a very short time; after a few minutes a series of changes begins to take place, by which the outer segments of both rods and cones become split into discs, and finally disintegrate so as to be altogether unrecognisable, even if not totally destroyed. Further, in an equally short time the nerve-fibres become varicose, and appear to be thickly studded with spindle-shaped knots; and other post-mortem changes rapidly occur. If, however, a fresh piece of retina be treated with a strong solution of osmic acid, the whole of the rods and cones will be found perfectly preserved after twenty-four hours' time, and the nerve-fibres will be found not to be varicose. After this preliminary hardening, portions of the retina may be treated with water (which would be ruinous to the structures of a fresh retina), they may even remain in water for days without harm; they may be stained, acidified, hardened, imbedded, cut into sections, and mounted in either aqueous or resinous media without suffering.

This example shows that one of the objects aimed at in fixing is to impart to tissues the degree of *hardening* necessary to enable them to offer such mechanical resistance to *post-mortem* change and to the processes of after-treatment as not to suffer change of form. Another important function of fixing is to *render insoluble* elements of cells and tissues that would otherwise be more or less dissolved out by the liquids employed in the after-treatment. A third and highly important function of fixing-agents consists in producing *optical differentiation* in structures. By coagulating the elements of tissues and cells, fixing agents *alter their indices of refraction*, raising them in varying degrees. They do not act in an equal degree on all the constituent elements of cells and tissues, but raise the index of some more than that of others, thus producing optical differentiation where there was little or none before. Compare the aspect of the epithelium of the tail of a living tadpole, observed in water, with its aspect after the action of a little diluted solution of Flemming. In the living state the protoplasm of its cells has a refractive index little superior to that of water, and consequently so low an index of visibility that hardly any structure can be made out in the object. But as soon as the protoplasm has been sufficiently coagulated by the reagent the refractive indices of some of its elements will have been raised to above that of balsam, the chromatin of the nuclei will be brought out, and other structures be revealed where none was visible before.

29. **The Action of Fixing Agents** consists in *coagulating* and rendering insoluble certain of the constituents of tissues. This is effected sometimes without any chemical action being involved, as when alcohol is employed, which acts by simple withdrawal of the water of the tissues. But in the majority of cases the fixing agents enter into *chemical combination* with certain of the elements of the tissues. The compounds thus formed are sometimes *unstable and soluble*, so that they are removable by washing, as is the case with several of those formed by picric acid. It is found in practice, however, that those formed by chromic acid and its salts, and the salts of the heavy metals, as mercury, iron, platinum, gold, and silver, are *mostly insoluble.*

The insolubility of these bodies is an advantage in that it ensures that the tissues shall not be robbed of their essential constituents, nor deprived of their desired consistency and optical differentiation, by the reagents subsequently employed. It is also sometimes an advantage in that certain of the compounds in question have the property of combining with certain colouring matters, and thus *affording important stains* which could not otherwise be obtained; or in other words, of acting as mordants.

But it is sometimes a disadvantage, inasmuch as these same compounds which render possible the production of some stains are hindrances to the production of others. Tissues that have been fixed with osmic or chromic acid or its salts are in general not easily to be stained with carmine or similar colouring matters, unless the metals have been previously removed by special chemical treatment; though they may generally be stained with hæmalum, or, after sectioning, with iron hæmatoxylin or tar colours.

According to Fischer (*Fixirung, Färbung, und Bau des Protoplasmas*, Jena, G. Fischer, 1899), the coagulation which constitutes fixation is, in the case of the liquid and semi-liquid constituents of tissues, *always* a phenomenon of *precipitation*. The more solid constituents (such as fibrils that are visible during life, nucleoli, and the like) he admits may be acted on by fixing reagents without the formation of any visible precipitates. But all the liquid ones, in so far as they are fixed at all, are visibly precipitated in special *precipitation forms*, which vary according to the precipitant. Each fixing agent gives its own characteristic *fixation image*, which may be more or less lifelike, but can never be absolutely so. Fischer gives copious descriptions of the precipitation forms of the chief organic compounds found in tissues, and of the precipitation powers of the chief fixing agents, which the reader will do well to study.

It seems to be a consequence of Fischer's theory of fixation by precipitation that the most energetic fixing agents should always be found amongst the most energetic precipitants. But on the showing of his experiments this is not so. For instance, it is allowed on all hands that osmic acid is a most energetic fixative. But Fischer finds (*op. cit.* pp. 12—14, 27) that it is a very incomplete and weak precipitant. Or, to take a contrary instance, he finds that picric acid is an energetic precipitant of the majority of cell constituents; but surely every cytologist must admit that it is not a highly energetic fixative.

It would seem to follow, from these instances and from other similar ones, that Fischer's tables of precipitating power cannot be taken as a measure of the fixing power of the reagents. And further, the study of the fixation images of tissues afforded by osmic acid, formaldehyde, and other reagents, seems to show that the coagulation brought about by them is in part accompanied by the formation of visible precipitates, but in part not so, and that they may do their work to a larger extent than he seems to admit through a *homogeneous coagulation*. But from his very suggestive observations it certainly appears that the formation of visible precipitates is a very wide-spread, if not universal concomitant of fixation; and that the wider the precipitating power of a fixative (*i.e.* the greater the number of organic liquids that it can precipitate), the greater will be the *number of artefacts* to which it can give rise.

30. The Characters of the Usual Fixing Agents.—A good fixing agent should first of all *preserve* all the elements it is desired to fix. But that is not enough; it should also give good optical *differentiation*, and should have sufficient power of *penetration* to ensure that small pieces of tissue be equally fixed by it throughout. No single substance or chemical compound fulfils all that is required of a good fixing agent; hence it is that all the best fixing agents are *mixtures*. Osmic acid, for instance, fulfils some of these conditions, but not all of them. It kills rapidly and preserves admirably the elements of cytoplasm, but nuclei not so well. But the optical differentiation that it gives, though sometimes good, is often very inferior. For osmic acid, by coagulating in nearly equal degrees alike the spongioplasm (the plastin reticulum) and the hyaloplasm (the enchylema) of the cell-body, and the chromatin of nuclei, raises alike the refractive indices of all of them; so that if the fixing action have been in the least degree overdone, the cells acquire a homogeneous aspect in which the finer details are obscured by the general refractivity of the whole. If now, instead of using it pure, it be used in combination with acetic acid, a better differentiation is obtained; for acetic acid, whilst enhancing, or at all events not interfering with the fixation of chromatin, serves to facilitate penetration and to counteract the excessive action of the osmic acid on the protoplasm, so that the cells come out less homogeneous and with more detail observable in them. A still better effect is obtained if to the osmic acid there be added not only acetic acid, but also chromic acid. For osmic acid has the property of blackening tissues, thus rendering them opaque. Chromic acid counteracts in a considerable degree this blackening action. It also helps probably to bring out the chomatin of nuclei, which is insufficiently fixed by the other two ingredients, and probably also helps in other ways to bring about optical differentiation; so that in the result a much clearer picture is obtained.

I take it that it has been established by experience that, *as a general rule*, in order to get the *most complete fixation*, fixatives should have an *acid reaction*. Consequently, if they have not naturally an acid reaction, they should be acidified, *e.g.* osmic acid should be acidified with acetic acid or the like. As a matter of fact, it will be found that acetic acid is very largely employed in mixtures. It is generally held that it acts in them as an agent for facilitating penetration and producing differentiation, as explained above, and also for ensuring the fixation of nuclein (if the other ingredients are not adequate thereto); but this is probably not all. FISCHER (in the work quoted above, pp. 10, 27, and other places) holds that its function in these mixtures is chiefly that of a *neutraliser* or *acidifier* (Ansäurer) for ensuring that the other ingredients shall have an acid, or at least a neutral medium to do their work in. For the precipitating power, that is, in his view, the fixing power of a reagent, varies according to the reaction, acid or alkaline, of the things to be fixed; and a feebly acid reaction is the one most favourable for ensuring precipitation. Many things that are quite unprecipitable by certain reagents whilst in alkaline or neutral solution are immediately precipitated by them if the solution is rendered acid. "Many kinds of cell contents," he says (*op. cit.*, p. 10) "indeed the majority, have an alkaline reaction, and are thereby quite inaccessible to the precipitating action of certain agents, such as osmic acid, or bichromate; and the action of certain other fixatives, such as platinum chloride and chromic acid, is more or less hindered by the presence of free alkalies. For neither the chromic acid (of solution of Flemming) nor the platinum chloride (of solution of Hermann) would be adequate to act as acidifiers to the osmic acid of the mixtures. They cannot do so, first, because they themselves become combined with the tissues much more quickly than the slowly working osmic acid, and secondly, because they themselves have only an extremely weak acid reaction." Hence the function of the organic acid is to bring into play the precipitating power of the other ingredients.

In default of special reasons to the contrary, fixing mixtures may conveniently be made to contain from 1 to 5 per cent. of acetic acid. But for some purposes they should be neutral, or even alkaline. See, for instance, *Neurofibrils*.

I think *the beginner* should avoid such things as liquid of FLEMMING and similar mixtures. He may take, instead, BOUIN'S picro-formol.

Corrosive sublimate (acidified) is a good all-round fixative, with excellent penetration, but is not quite so easy to manage.

Picric acid gives a fair though weak fixation, with very good penetration, is easy to manage, and does not make tissues brittle, which sublimate easily may do. Pure formol is not bad, and very easy to manage.

Speaking generally, osmic acid, chromic acid, bichromates, chloride of platinum, and the majority of the compounds of the heavy metals, are *hindrances* to staining; whilst heat, alcohol, trichloracetic acid, formol, corrosive sublimate, nitric acid, picric acid, and acetic acid, are *neutral*, or even *favourable*, in this respect.

31. The Practice of Fixation.—See that the structures are *perfectly living* at the instant of fixation, otherwise you will only fix pathological states or post-mortem states.

Fixation is generally performed by *immersion* of the objects in the fixing liquid. In this case, everything should be done to facilitate the *rapid penetration* of the fixing agent. To this end let the structures be divided into the smallest portions that can conveniently be employed, and if entire organs or organisms are to be fixed whole, let openings, as large as possible, be first made in them.

The penetration of reagents is greatly facilitated by *heat*. You may warm the reagent and put it with the object to be fixed in the paraffin stove, or you may even employ a fixing agent heated to boiling-point (as boiling sublimate solution for certain corals and Hydroids, or boiling absolute alcohol for certain Arthropods with very resistent integuments). But this should only be done as a last resource.

Let the *quantity* of fixing agent employed be at least *many times* the volume of the objects to be fixed. If this precaution be not observed the composition of the fixing liquid may be seriously altered by admixture of the liquids or of the soluble substances of the tissues thrown into it. For a weak and slowly acting fixing agent, such as picric acid, the quantity of liquid employed should be in volume about one hundred times that of the object to be fixed. Reagents that act very energetically, such as Flemming's solution, may be employed in smaller proportions.

But fixation may also be performed by *injection* of the fixing liquid into the objects, thus ensuring a more rapid and thorough penetration of *voluminous* objects. See for this practice the methods of fixation by injection of GOLGI, DE QUERVAIN, MANN, and others, given under *Nervous System.*

BRAUS and DRUENER (*Jena. Zeit. Naturw.*, Bd. xxix, 1895, p. 435) fix fishes *by injection* through the *bulbus aortæ.* The vessels are first washed out with normal salt solution, and the fixing liquid is then thrown in.

KOLMER (*Anat. Anz.*, xlii, 1912, p. 47) fixes thus even large mammals (Chimpanzee, Goat). He first washes out with RINGER'S solution.

It is well not to leave specimens in fixing liquids longer than is sufficient to obtain the desired reaction. Sublimate, for instance, soon makes tissues brittle. But long immersion may be necessary to produce the desired optical differentiation with some reagents.

Careful *washing out* (by which is meant the removal from the tissues of the excess of uncombined fixative) is necessary in order to get tissues to stain properly. But it is not always equally imperative. Alcohol and formaldehyde do not require washing out before staining ; acetic and picric acid only for some stains ; sublimate will allow of staining even if not washed out, but allows of a sharper stain if well washed out ; all osmic, chromic, and platinic liquids require very thorough washing out.

It is important to use the *appropriate liquid for washing out* the fixing agent after fixation. It is frequently by no means a matter of indifference whether water or alcohol be employed for washing out. Sometimes water will undo the whole work of fixation (as with picric acid). Sometimes alcohol causes precipitates that may ruin the preparations. Objects fixed in alcohol, formol, acetic acid, picric acid, or nitric acid require to be washed out with alcohol, or at least with some hardening liquid, whilst those that have been fixed with osmic or chromic acid, or with one of the other compounds of the heavy metals, require *in general* to be washed out with water. Sublimate, however, is best washed out with alcohol.

Use *liberal quantities* of liquid for washing.

Change the liquid as often as it becomes turbid, if that should happen.

The process of washing out is greatly facilitated by *heat*. Picric acid, for instance, is nearly twice as soluble in alcohol warmed to 40° C. as in alcohol at the normal temperature (Fol).

0 4 Fol.

32. Fixation of Marine Animals.—The tissues of *marine organisms* are as a general rule more refractory to the action of reagents than those of corresponding fresh-water or terrestrial forms, and fixing solutions should in consequence be stronger (about two to three times).

Marine animals ought to be *freed from the sea water* adherent to their surface before treating them either with alcohol or any fixing reagent that precipitates the salts of sea water. If this be not done, the precipitated salts will form on the surfaces of the organisms a crust that prevents the penetration of reagents to the interior. Fixing solutions for marine organisms should therefore be such as serve to keep in a state of solution, and finally remove, the salts in question. If alcohol be employed, it should be *acidified* with hydrochloric or some other appropriate acid. Picro-nitric acid is a fixing reagent that fulfils the conditions here spoken of. (On this subject see MAYER, in *Mitth. Zool. Stat. Neapel*, ii (1881), p. 1, *et seq.*)

33. Hardening.—The process of hardening is distinguished from that of fixing as being directed to the attainment of a degree of consistency sufficient to allow of soft tissues being cut into sections without imbedding. It is an *after-process*, and only ranks as a *special* method.

Methods of imbedding have now been brought to such a degree of perfection that the thorough hardening of soft tissues that was formerly necessary in order to cut thin sections from them is, in the majority of cases, no longer necessary. But there are some exceptions. Such are, for instance, the cases in which it is desired to cut very large sections, such as sections of the entire human brain.

The reagents employed for hardening are for the most part of the same nature as those employed for fixing. But it does not follow that all fixing agents can be employed for hardening. Corrosive sublimate, for instance, would be most inappropriate as a hardening agent.

34. The Practice of Hardening.—Employ *in general* a *relatively large volume* of hardening liquid, and change it very frequently. If the volume of liquid be insufficient, its composition will soon became seriously altered by the diffusion into it of the soluble substances of the tissues; and the result may be a macerating instead of a hardening liquid. Further, as soon as, in consequence of this diffusion, the liquid has acquired a composition similar in respect of the proportions of colloids and crystalloids contained in it to that of the liquids of the tissues, osmotic equilibrium will become established, and diffusion will cease; that is to say, the hardening liquid will cease to penetrate. This means, of course, maceration of internal parts. On the other hand, it appears that a certain slight proportion of colloids in the hardening liquid is favourable to the desired reaction, as it gives a better consistency to the tissues by preventing them from becoming brittle. Hence the utility of employing *a certain proportion* of hardening agent.

Hardening had better be done in tall cylindrical vessels, the objects being suspended by a thread, or muslin bag, or otherwise, at the top of the liquid. This has the advantage of allowing diffusion to take place as freely as possible, whilst any precipitates that may form fall harmlessly to the bottom; or, they may be laid on a layer of cotton-wool, or filter-paper, or spun glass.

In general, *begin* hardening with a *weak reagent*, increasing the strength gradually, as fast as the tissues acquire a consistence that enables them to support a more energetic action of the reagent.

Let the objects be removed from the hardening fluid as soon as they have acquired the desired consistency.

CHAPTER IV.

FIXING AND HARDENING AGENTS—MINERAL ACIDS AND THEIR SALTS.

35. Osmic Acid.—The tetroxide of osmium (OsO_4) is the substance commonly known as osmic acid, though it does not possess acid properties. It is extremely volatile, and in the form of an aqueous solution become partially reduced with great readiness in presence of the slightest contaminating particle of organic matter. It is generally believed that the aqueous solutions are reduced by light alone, but this is not the case: they may be exposed to the light with impunity *if dust be absolutely denied access to them.*

The solution of osmic acid in chromic acid solution is not, like the solution in pure water, easily reducible, but may be kept without any special precautions. I therefore keep the bulk of my osmium in the shape of a 2 per cent. solution of osmic acid in 1 per cent. aqueous chromic acid solution. This solution serves for fixation by osmium vapours, and for making up solution of Flemming, which is the form in which osmium is most generally employed. A small quantity of osmic acid may also be made up in 1 per cent. solution in distilled water, and kept in a drop-bottle with grooved stopper, from which quantities can be obtained when required without removing the stopper.

Cori (*Zeit. wiss. Mik.*, vi, 1890, p. 442) finds that solutions in distilled water keep perfectly if there be added to them enough permanganate of potassium to give a very slight rosy tint to the liquid. From time to time, as the solution becomes colourless, further small quantities of the salt should be added, so as to keep up the rosy tint.

Busch finds that the addition of sodium iodate hinders reduction (*Neurol. Centralb.*, xvii, 1898, p. 476).

Pintner finds that a slight addition of corrosive sublimate has the same effect, *e.g.* ten drops of 5 per cent. solution of sublimate added to 100 c.c. of 1 per cent. solution of osmic acid.

For the so-called "regeneration" of reduced solutions, see *previous editions*.

Osmic acid is found in commerce in the solid form in sealed tubes. The assigned weights should be checked, as they may vary greatly (this does not apply to the tubes sent out by Grübler and Hollborn, or Kahlbaum).

Fixation by the Vapours.—This is indicated in most of the cases in which it is possible to expose the tissues directly to the action of the vapour. The tissues are pinned out on a cork, which must fit well into a wide-mouthed bottle in which is contained a little solid osmic acid (or 1 per cent. solution will do). Very small objects, such as isolated cells, are simply placed on a slide, which is inverted over the mouth of the bottle. They remain there until they begin to turn brown (isolated cells will generally be found to be sufficiently fixed in thirty seconds: whilst in order to fix the deeper layers of relatively thick objects, such as retina, an exposure of several hours may be desirable). It is well to wash the objects with water before staining, but a very slight washing will suffice. For staining, methyl-green may be recommended for objects destined for study in an aqueous medium, and, for permanent preparations, alum-carmine, picrocarmine, or hæmatoxylin.

In researches on nuclei, it may be useful to employ the vapours of a freshly prepared mixture of osmic and formic or acetic acid (Gilson, *La Cellule*, i, 1885, p. 96).

An apparatus for conveniently employing the vapours is described by ANDREWS (*Zeit. wiss. Mik.*, xiv, 1898, p. 448).

The reasons for preferring fixation by the vapour are that osmic acid is more highly penetrating in vapour than in solution; that the arduous washing out required by the solutions is done away with; and that all possibility of deformation through osmosis is eliminated.

Fixation by Solutions.—Osmic acid is now very seldom used *pure* in the shape of solutions. When, however, it is so employed it is used in strengths varying from $\frac{1}{20}$ per cent. to 1 per cent. I should say myself that, as a rule, not more than 0·1 per cent., and *never more than* 0·5 per cent., should be used.

On account of its feeble penetrating power the objects to be fixed should be *as small as possible.*

A little acetic or formic acid (0·5 to 1 per cent.) may generally with advantage be added to the solutions just before using.

The solutions should be kept protected from the light during the immersion of tissues. (This precaution is not necessary if Flemming's or Hermann's solution be used). If the immersion is to be a long one the tissues must be placed with the solution in well-closed vessels. The objects may be deemed to be fixed as soon as they have become brown throughout.

After-Treatment.—The excess of osmic acid must be well washed out before proceeding to any further steps in preparation; water should be used for washing. Notwithstanding the greatest care in soaking, it frequently happens that some of the acid remains in the tissues, and causes them to *blacken* in time, and in any case hinders staining. To obviate this blackening it has been advised to wash them out in ammonia-carmine or picro-carmine (not very effectual), or to soak them for twenty-four hours in a solution of bichromate of potash (Müller's solution or Erlicki's will do), or in 0·5 per cent. solution of chromic acid, or in Merkel's solution. The treatment with bichromate solutions has the great advantage of highly facilitating staining with carmine or hæmatoxylin. Max Schultze recommended washing, and mounting permanently in acetate of potash. Fol, treatment with a weak solution of carbonate of ammonia. But the best plan of all is to properly *bleach* the preparations (see "**Bleaching**"). This may be done by means of *peroxide of hydrogen.* Overton (*Zeit. wiss. Mik.*, vii, 1890, p. 10) finds that it is completed in a few minutes in a mixture of 1 part commercial peroxide with 10 to 25 parts 70 per cent. alcohol. (The commercial peroxide, slightly acidulated with HCl, will keep well in the dark; but the mixture with alcohol must be made fresh for use.) According to Bristol (*Amer. Natural*, xxvii, 1893, p. 176) the peroxide acts best in the sun. Binet (*Journ. de l'Anat. et de la Physiol.*, xxx, 1894, p. 449) has successfully used permanganate of potash.

Mann (*Methods,* etc., p. 83) takes a solution of 0·25 per cent., and treats the browned tissues with 1 part of saturated solution of sulphurous acid to 9 of normal salt solution. . . . Mönckeberg and Bethe (*Arch. Mik. Anat.,* liv, 1899, p. 135) have succeeded in satisfactorily restoring the staining susceptibility of osmium material by means of sulphurous acid (obtained by adding hydrochloric acid to bisulphite of sodium, 2 to 4 drops of the acid added to 10 c.c. of a 2 per cent. solution of the salt).

Fol (*Lehrb.,* p. 174) recommends a weak aqueous solution of ferricyanide of potassium.

I find the sulphate of iron solution used in Benda's hæmatoxylin stain has a marked bleaching effect, and so also, though in a less degree, the iron alum of Heidenhain's process.

Altmann (*Die Elementarorganismen,* pp. 33 and 35) puts sections overnight into gold chloride of 2 per cent., and reduces in formic acid in the sun, and removes the gold by iodised alcohol.

But perhaps the best plan is the chlorine method of Mayer, or his magnesium peroxide, for both of which see *Bleaching.*

The same stains recommended for objects fixed by the vapours will be found useful here. For sections, of course, in both cases safranin and other anilin stains may be employed with advantage, as may hæmatoxylin.

In general osmic acid, especially when used in the form of vapour, fixes protoplasm very faithfully, nuclei badly. It is pre-eminently a fixative of the *hyaloplasm* or enchylema of cells. The *penetrating power* of the solution is *very low,* so that if any but very small pieces of tissue be taken the outer layers become over-fixed before the reagent has penetrated to the deeper layers. Over-fixed cells have a certain homogeneous, glassy, or colloid look, and are unfit for study, and attention should be confined to cells four or five layers deeper down, which will generally be found to present the required intensity of fixation. In these the fixation is admirable, with no shrinkage and next to no swelling of anything.

36. Osmic Mixtures.—Ranvier et Vignal (Ranvier, *Leç. d'Anat. Gén.,* p. 76; Vignal, *Arch. de Physiol.,* 1884, p. 181) take equal volumes of 1 per cent. osmic acid and 90 per cent. alcohol (*freshly mixed*). They wash out in 80 per cent. alcohol, then wash with water and stain for forty-eight hours in picro-carmine or hæmatoxylin.

NICOLAS (*Intern. Monatsschr.*, 1891, p. 3) adds ½ per cent. of osmic acid to nitric acid of 3 per cent. I have employed a similar mixture and not had good results, though I find the mixture keeps perfectly.

BUSCH (*Neurol. Centralb.*, xvii, 1898, No. 10, p. 476; *Zeit. wiss. Mik.*, xv, p. 373) finds that the penetration of osmic acid is enhanced by combining it with iodate of sodium, which by hindering its too rapid decomposition in the tissues ensures a more energetic action in the deeper layers. He adds 3 per cent. of sodium iodate to a 1 per cent. solution of osmic acid.

UNNA (*Monatschr. prakt. Derm.*, xxvi, 1898, p. 602) adds 1 per cent. of alum to a 1 per cent. solution. For some mixtures of KOLOSSOW, see *5th ed.*, or *Zeit. wiss. Mikr.*, v, 1888, p. 51, and ix, 1892, p. 39.

37. Chromic Acid.—Chromic anhydride, CrO_3, is found in commerce in the form of red crystals that dissolve readily in water, forming chromic acid, H_2CrO_4. These crystals are very deliquescent, and it is therefore well to keep the acid in stock in the shape of a 1 per cent. solution. Care must be taken not to allow the crystals to be contaminated by organic matter, in the presence of which the anhydride is readily reduced into sesquioxide.

Chromic acid is generally employed in aqueous solution. Some observers (KLEIN; URBAN PRITCHARD; PERÉNYI) have recommended alcoholic solutions; but this is evidently irrational. For in the presence of alcohol chromic acid has a great tendency to *become reduced* to chromous oxide or sesquioxide, neither of which appears to have any fixing power.

The most useful strengths in which it is employed in aqueous solution are from 0·1 to 1·0 per cent. for a period of immersion of a few hours (structure of cells and ova). For nerve tissues weaker solutions are taken, $\frac{1}{50}$ to $\frac{1}{8}$ per cent. for a few hours. Stronger solutions, such as 5 per cent., should only be allowed to act for a few seconds.

Washing out.—The general practice is to wash out very thoroughly with water (by preference running water, for many hours), before bringing into alcohol or any staining liquid. For if the objects are put direct into alcohol it is found that after a short time a fine precipitate is thrown down on the surface of the preparations, thus forming an obstacle to the further penetration of the alcohol. Previous washing by water does not prevent the formation of this precipitate, and changing the alcohol does not prevent it from forming again and again. It has, however, been found by Hans Virchow (*Arch. mik. Anat.*, xxiv, 1885, p. 117) that it may be entirely prevented by simply keeping the preparations *in the dark*. The alcohol becomes yellow as usual (and should be changed as often as this takes place), but no precipitate is formed. If this precaution be taken, previous washing with water may be omitted, or at all events greatly abridged.

Mayer (*Grundzüge*, 1st ed., p. 28) proceeds as follows:—The fixed material is merely rinsed in water and brought direct into 70 per cent. alcohol. It is washed therein, preferably in the dark, until after several changes the alcohol remains colourless. It is then either passed through higher alcohols and imbedded in paraffin, the chromous oxide (or whatever chrome compound it may be that is present in the tissues) being removed from *the sections* after these are made; or this necessary removal is performed at once. If this be preferred, the material is brought into sulphuric acid diluted with twenty volumes of water, or into nitric acid diluted with ten volumes of water. After at most a few hours therein, it will have become of a light greyish green, and on removal of the acid may be readily stained. If it be preferred to treat *the sections*, it is sufficient to put them into the usual hydrochloric acid alcohol (four to six drops of HCl to 100 c.c. of 70 per cent. alcohol), in which after a short time they become almost white, and will stain excellently with any of the usual stains. So also Edinger (*Zeit. wiss. Mik.*, i, 1884, p. 126; nitric acid 1 : 20 for five minutes). Unna (*Arch. mik. Anat.*, xxx, 1887, p. 47) holds that the chrome is present in the tissues in the form of chromic chromate, and removes it by treatment with peroxide of hydrogen. Overton (*Zeit. wiss. Mik.*, vii, 1890, p. 9) employs a weak solution of sulphurous acid, which converts it into a sulphate. See also the directions for bleaching osmic acid preparations, § 35.

Tissues that have been fixed in chromic acid may be stained in aqueous solutions, as water does not have an injurious effect on them.

The best stain for chromic material that has not been treated by Mayer's special process, or by a similar one, is hæmatoxylin, or, for sections, the basic tar colours.

Chromic acid is not a very penetrating reagent, and for this reason, as well as for others, is now seldom used *pure* for *fixing*.

For prolonged hardening it is generally employed in strengths of 1/5 per cent. to 1/2 per cent., the immersion lasting a few days or a few weeks, according to the size and nature of the object. Mucous membrane, for instance, will harden satisfactorily in a few days; brain will require some six weeks.

Large quantities of the solution must be taken (at least 200 grammes for a piece of tissue of 1 centimetre cube—Ranvier).

In order to obtain the best results you should not employ portions of tissue of more than an inch cube. For a human spinal cord you should take two litres of solution, and change it for fresh after a few days. Six weeks or two months are necessary to complete the hardening.

I think it is frequently useful to *add a little glycerin*; there is less brittleness.

The solution should be taken weak at first, and the strength increased after a time. The objects should be removed from the solution as soon as they have acquired the desired consistency, as if left too long they will become brittle. They may be preserved till wanted in alcohol (95 per cent.). It is well to wash them out in water for twenty-four or forty-eight hours before putting them into the alcohol. After a time they generally become green in the alcohol. They may be *bleached* if desired.

Chromic acid is a most powerful and rapid hardening agent. (By it you may obtain in a few days a degree of hardening that you would hardly obtain in as many weeks with bichromate, for instance.) It has the defect of a great tendency to cause *brittleness*.

38. **Chromic Acid and Alcohol** (URBAN PRITCHARD, *Quart. Journ. Mic. Sci.*, 1873, p. 427).—Chromic acid, 1 part; water, 20 parts; rectified spirit, 180 parts. For hardening such tissues as retina, cochlea, etc.

A mixture of 2 parts of 1/6 per cent. chromic acid solution with 1 part of methylated spirit was once much used by KLEIN (*Quart. Journ. Mic. Sci.*, 1878, p. 315).

Both these mixtures are irrational (see § 37). A still more irrational mixture with oxalic acid has been propounded by GRAF (*Cont. Path. Inst. New York State Hosp.*, 1898, No. 15; see *Grundzüge*, 4th ed., p. 34).

39. Chromo-acetic Acid (FLEMMING, *Zellsbz., Kern. u. Zellth.*, p. 382).

Chromic acid . . 0·2 to 0·25 per cent.
Acetic acid . . 0·1 per cent. in water.

Flemming found this the best reagent for the study of the *achromatic* elements of karyokinesis. You can stain with hæmatoxylin, or the basic anilin dyes.

The following has been recommended for Annelids by EHLERS:—To 100 c.c. of chromic acid of 0·5 to 1 per cent. add from 1 to 5 drops of glacial acetic acid. The acetic acid is said to be sufficient to counteract any shrinkage due to the chromic acid.

Similar to this is the "chromo-acetic acid, No. 1," of LO BIANCO (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443), viz. 1 part 50 per cent. acetic acid and 20 parts 1 per cent. chromic acid, which is found very useful for fixing marine animals.

40. Chromo-formic Acid (RABL, *Morph. Jahrb.*, x, 1884, pp. 215, 216).—Four or five drops of concentrated formic acid are added to 200 c.c. of 0·33 per cent. chromic acid solution. The mixture must be freshly prepared at the instant of using. Fix for twelve to twenty-four hours, wash out with water. Used by Rabl for the study of karyokinesis.

41. Chromo-osmic Acid (MAX FLESCH, *Arch. mik. Anat.*, xvi, 1879, p. 300).—Osmic acid. 0·10; chromic acid, 0·25; water, 100·0. LO BIANCO (*Mitth. Zool. Stat. Neapel.*, ix, 1890, p. 443) employs for marine animals a mixture of 1 part 1 per cent. osmic acid and 50 parts 1 per cent. chromic acid.

42. Chromo-aceto-osmic Acid (FLEMMING, *Zellsubstanz, Kern und Zelltheilung*, 1882, p. 381). FIRST or WEAK formula:

$$\left.\begin{array}{lll} \text{Chromic acid.} & . & 0{\cdot}25 \text{ per cent.} \\ \text{Osmic acid} & . & 0{\cdot}1 \quad ,, \\ \text{Glacial acetic acid} & . & 0{\cdot}1 \quad ,, \end{array}\right\} \text{in water.}$$

MEVES (*Encycl. mikr. Techn.*, 1, p. 475) sometimes adds 1 per cent. of sodium chloride.

Fol (*Lehrb. d. vergl. mik. Anat.*, 1884, p. 100) recommends the following variant:

1 per cent. chromic acid	25 vols.
1 per cent. osmic acid	2 ,,
2 per cent. acetic acid	5 ,,
Water	68 ,,

—that is to say, a mixture much weaker in osmium than Flemming's.

A mixture still weaker than this in osmium, viz. with 1 vol. osmic acid solution, instead of 2, has been recommended by Cori (*Zeit. wiss. Mik.*, vi, 1890, p. 441).

Second or Strong formula (*Zeit. wiss. Mik.*, 1, 1884, p. 349):

1 per cent. chromic acid	15 parts.
2 per cent. osmic acid	4 ,,
Glacial acetic acid	1 part.

If this mixture be kept in stock in large quantities, it may go bad, on account of the large proportion of organic acid contained in it. I therefore recommend that the osmic and chromic acid be kept ready mixed in the proportions given, and 5 per cent. of acetic acid added at the moment of using.

Weaker Formula.—More recently, Flemming has been making up the mixture with only 2 parts of the osmic acid instead of 4, and has spoken of this modification as "weaker osmium mixture" (Meves, in *Encycl. Mikr. Techn.*, p. 476).

Meves (*loc. cit.*) takes for *delicate objects* 15 parts of chromic acid of only 0·5 per cent., 2 or 4 of osmic acid of 2 per cent., and 1 of acetic acid, and thus gets less shrinkage.

Podwyssozki recommends (for glands especially) the following modification:

1 per cent. CrO_3 dissolved in 0·5 per cent. solution of corrosive sublimate	15 c.c.
2 per cent. osmic acid solution	4 c.c.
Glacial acetic acid	6 to 8 drops.

The sublimate is said to augment the penetration of the osmium, but is unfavourable to staining (Ziegler's *Beiträge z. path. Anat*, i, 1886; *Zeit. wiss. Mik.*, iii, 1886, p. 405).

The first or weak liquid is the better for very small objects, the second or strong one for larger ones, as it has *better penetration*. These liquids may be allowed to act for many hours or days, or according to some workers even weeks or months; but this exaggerated fixation is clearly only justifiable in very special cases, if at all. Wash out very thoroughly in water (running, 24 hours, or treat as directed for chromic acid § 37). Stain with alum hæmatoxylin if you wish to stain *in toto* (staining in this way with other reagents is possible, but difficult). Stain sections with safranin or other basic coal-tar colour, or with iron hæmatoxylin.

For fixing with the *strong* mixture you need only take a bulk of liquid of some 4 times the volume of the objects (but with the weak mixture the proportion should be increased). Both of them are first-rate fixatives of cellular structures, both as regards their *preservation* and as regards their *optical differentiation*. But they must be properly used, and not applied to objects for which they are not fitted. For instance, their *power of penetration* is *extremely bad*; they will not fix properly, even in a loose-celled tissue, through more than a layer of about five cells thick. They are therefore suitable only for *very small* objects or for *very small* pieces of tissue, such as suffice for cytological or histological work. The strong liquid especially has not the character of a *general* reagent. As a matter of fact it was recommended by Flemming in the first instance merely for a very special purpose, the hunting for karyokinetic figures, and not for general purposes. It is still very much used, but in my opinion unadvisedly. In most cases, Bouin's picroformol will do all that it is intended to do, without its disadvantages.

It may be used for prolonged hardening, *e. g.* of small pieces of nervous tissue, and is very good for that purpose.

Fat is blackened (or browned) by it (see § 35). Chromatin is mordanted by it for basic anilin dyes, enabling them to give peculiarly sharp and powerful stains.

43. Osmic Acid and Bichromate.—Altmann (*Die Elementarorganismen*, Leipzig, 1890), takes for his "bioblasts" a mixture of equal parts of 5 per cent. solution of bichromate of potash and 2 per cent. solution of osmic acid. The bichromate ought not to contain any free chromic acid.

Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) employs for marine animals a mixture of 100 c.c. of 5 per cent. solution of bichromate and 2 c.c. of 1 per cent. osmic acid.

Hoehl (*Arch. Anat. Phys., Anat. Abth.*, 1896, p. 31), recommends a mixture of 80 c.c. of 3 per cent. bichromate, 20 c.c. of 1 per cent. osmic acid, and 2 c.c. of glacial acetic acid.

44. Osmic, Bichromate, and Platinic Mixture (Lindsay Johnson's Mixture).—Latest formula, 1895, communicated by Dr. Lindsay Johnson :

Bichromate of potash (2·5 per cent.) .	70 parts.
Osmic acid (2 per cent.) . . .	10 "
Platinum chloride (1 per cent.) .	15 "
Acetic or formic acid . . .	5 "

Henneguy, who has worked a great deal with this reagent, and recommends it highly, says (*Leçons sur la Cellule*, p. 61) that it is well only to add the acetic or formic acid just before using, as it frequently reduces the osmium and platinum very rapidly and energetically. He finds that it contracts the more spongy sorts of protoplasm less than mixture of Flemming. I think highly of it—for certain objects.

45. Platino-aceto-osmic Acid (Hermann's solution (*Arch. mik. Anat.*, xxxiv, 1889, p. 58). One per cent. platinum chloride 15 parts, glacial acetic acid one part, and 2 per cent. osmic acid either 4 parts or only 2 parts. Hermann found that protoplasmic structures are thus better preserved than with the chromic mixture.

The after-treatment and staining should be the same as for objects treated with Flemming's solution. Rengel (*Zeit. wiss. Zool.*, lxiii, 1898, p. 454) washes out for half an hour to an hour with saturated aqueous sol. of picric acid, which he thinks facilitates the staining, especially of nuclei.

The action of this fixative is, roughly, similar to that of Flemming's. Like Flemming's, it mordants chromatin for staining with "basic" colours, with which it affords equally fine nuclear stains. But, owing to the platinum in it, it diminishes more than Flemming's the colorability of tissues with "acid" colours, so that it is *extremely difficult* to obtain *good plasma stains* after its action. It causes a notable shrinkage in chromatin. It gives a *full* fixation of cytoplasm, to which it gives a much more fine-grained aspect than liquid of Flemming does.

A mixture of BORREL'S (from CAULLERY and MESNIL, *Arch. Protistenk*, vi, 1905, p. 281), consists of 2 parts of osmic acid, 2 of platinum chloride, 3 of chromic acid, 20 of acetic acid, and 350 of water.

46. **Rawitz** (*Zeit. wiss. Mikr.*, xxv, 1909, p. 386) takes 4 parts of Kahlbaum's **Phospho-Tungstic** acid, 5 of alcohol, and 1 of acetic acid, added just before use, fixes for 24 hours, and washes out the sections before staining with water containing a little calcium acetate.

47. **Nitric Acid** (ALTMANN, *Arch. Anat. Phys*, 1881, p. 219)—Altmann employs for fixing embryos dilute nitric acid, containing from 3 to 3½ per cent. pure acid. Such a solution has a sp. gr. of about 1·02. Stronger solutions have been used, but do not give such good final results. After extensive trial I find ALTMANN'S solution to be a second-rate reagent, giving a weak and thin fixation.

HIS (*ibid.*, 1877, p. 115) recommended a 10 per cent. solution. Flemming at one time employed solutions of 40 to 50 per cent. for the ova of Invertebrates.

TELLYESNICZKY (*Arch. mik. Anat.*, lii, 2, 1898, p. 222) thinks that "for general cell-fixing" the proper strength is 2 per cent. to 2½ per cent., as stronger grades act too energetically on the superficial layers.

MAYER has had good results with 5 per cent. solution.

Nitric acid has the valuable property of *hardening yolk without making it brittle.*

Pure water should in no case be used for washing out; the preparations should be brought direct into alcohol. Some persons take absolute, but I should say 70 per cent. is more generally indicated. Rabl has employed a 1 or 2 per cent. solution of alum.

For *prolonged hardening*, strengths of from 3 to 10 per cent. are sometimes employed. A strength of 12 per cent., allowed to act for two or three weeks, is said to afford very tough preparations of the encephalon.

BENDA (*Verh. Anat. Ges.*, 1888; *Ergeb. d. Anat.*, i, 1891, p. 7) fixes for twenty-four to forty-eight hours in 10 per cent. nitric acid, and then hardens in bichromate of potash.

Fol's Mixture (verbally communicated to me).—Three vols. of nitric acid, with 97 vols. of 70 per cent. alcohol.

48. **Chromo-nitric Acid** (PERÉNYI'S formula, *Zool. Anzeig.*, v, 1882, p. 459):

4 parts 10 per cent. nitric acid.
3 parts alcohol.
3 parts 0·5 per cent. chromic acid.

Fix for 4 to 5 hours and pass into alcohol of 70 per cent.

This mixture has been criticised (see previous editions) as irrational, the alcohol reducing the chromic acid and itself becoming etherised by the nitric acid. Some workers reject it, especially for ova, for which it is specially intended. But others speak highly of it. I myself have used it extensively for preparing objects for dissection and museum specimens, and found it admirable for these purposes. But preparations made to test its value from a cytological point of view have given me only second-rate results. It is now little used.

49. Chromic Acid and Platinum Chloride (MERKEL'S *Macula lutea des Menschen*, Leipzig, 1870, p. 19).—Equal volumes of 1·400 solution of chromic acid and 1·400 solution of platinum chloride. Objects should remain in it for several hours or even days. After washing out with alcohol of 50 per cent. to 70 per cent., objects stain excellently. If objects that have been fixed by osmic acid be put into it for some hours, blackening is said to be effectually prevented.

This is an excellent hardening medium for delicate objects. Merkel allowed from three to four days for the action of the fluid for the retina; for Annelids Eisig employs an immersion of three to five hours, and transfers to 70 per cent. alcohol; for small leeches Whitman finds one hour sufficient, and transfers to 50 per cent. alcohol.

A similar mixture, with the addition of 0·25 to 0·1 per cent. of acetic acid, is recommended by BRASS for Protozoa; and LAVDOWSKY has used for nuclei a mixture of 10 parts of 1 per cent. chromic acid, 5 of 1 per cent. platinum chloride, and 100 of 5 per cent. acetic acid.

Whitman recommends for the hardening of pelagic fish ova, a stronger mixture (due, I believe, to Eisig), viz.—

0·25 per cent. solution of platinum chloride .	1 vol.
1 per cent. solution of chromic acid . .	1 ,,

The ova to remain in it one or two days (WHITMAN, *Methods in Micro. Anat.*, p. 153).

Salts.

50. Chromates.—The chromates are amongst the oldest and best tried of hardening agents. The bichromate of potash especially was at one time universally employed for hardening all sorts of tissues.

FLEMMING (*Arch. mik. Anat.*, xviii, 1880, p. 352) pointed out that though it preserves cytoplasm well it causes chromatin to swell, and therefore should not be employed *for the study of nuclei*. But, *duly corrected with acetic acid*, it affords a correct and fine fixation of nuclei; whilst preserving *hyaloplasm and its inclusions,* secretions, etc., much better than chromic acid.

For an elaborate study of the action of chromic salts on nucleus and cytoplasm, see BURCKHARDT, *La Cellule,* xii, 1897, p. 335. He finds that the bichromates of sodium, ammonium, magnesium, strontium, and zinc have the same destructive action on nuclei that the bichromate of potassium has; but that the bichromates of barium, calcium, and copper have not. He concludes that *acetic acid ought always to be added,* not only to ensure the correct fixation of nuclei, but also to enhance penetration and the good preservation of cytoplasm. The following is recommended by him as a good combination for the fixation both of cytoplasm and nucleus:

Bichromate of barium, 4 per cent. solution .	60 vols.
Bichromate of potassium, 5 per cent. solution	30 ,,
Glacial acetic acid	5 ,,

(Instead of the barium you may take 4 per cent. solution of bichromate of calcium, or 6 per cent. solution of bichromate of copper.)

For the demonstration of the achromatic figure of cell division he recommends—

Chromic acid, 1 per cent. solution . .	60 vols.
Bichromate of potassium, 5 per cent. solution	30 ,,
Glacial acetic acid	5 ,,

51. Bichromate of Potash.—Perhaps the most important of all known *hardening* agents, *sensu stricto.* It hardens slowly, much more so than chromic acid, but it gives an incomparably better consistency to the tissues. They may remain almost indefinitely exposed to its action without much hurt.

The strength of the solutions employed is from 2 to 5 per cent. As with chromic acid, it is extremely important to begin with weak solutions and proceed gradually to stronger ones. About three weeks will be necessary for hardening a sheep's eye in solutions gradually raised from 2 to 4 per cent. Spinal cord requires from three to six weeks; a brain at least as many months.

After hardening, the objects should be well soaked out in water before being put into alcohol, or be treated as directed for chromic acid, § 37. They had better be kept in the dark when in alcohol (*see* § 37). (BÖHM and OPPEL [*Taschenbuch,* 3 Auf., 1896, p. 22] fix in the dark.) *If you wish to have a good stain with carmine you should not put the objects into alcohol at all, even for a second, until they have been stained.* You may stain either with carmine or hæmatoxylin, as well as with tar colours.

Bichromate objects have an ugly yellow colour which cannot be removed by mere soaking in water. It is said that it can be removed by washing for a few minutes in a 1 per cent. solution of chloral hydrate.

Prof. GILSON writes me that alcoholic solution of sulphurous anhydride (SO_2) is very convenient for the rapid decoloration of bichromate objects. A few drops suffice. *See* also § 37, and "Bleaching."

To facilitate staining with hæmatoxylin, WOLFF (*Zeit. wiss. Mik.*, xv, 3, 1899, p. 311) first stains in Boehmer's hæmatoxylin for twenty-four hours, and then for a few minutes in the same hæmatoxylin to which has been added 1 drop per watch-glassful of 5 per cent. solution of oxalic acid.

The simple aqueous solution of bichromate is hardly to be recommended as a *fixing* agent, because not only does it not preserve nuclei properly, but also because it penetrates very slowly. The first of these defects may be overcome entirely, the second to some extent by addition of acetic acid; whence the liquid of TELLYESNICZKY, next §.

52. Acetic Bichromate (TELLYESNICZKY, *Arch. mik. Anat.*, lii, 1889, p. 242)

Bichromate	3 grms.
Glacial acetic acid . . .	5 c.c.
Water	100 ,,

Smaller objects to remain in the fluid for one or two days, larger ones longer. Wash well in plenty of water, and pass through alcohols of increasing strength, beginning with 15 per cent.

Mixtures of bichromate with osmic acid have been given above, §§ 43 and 44.

53. MÜLLER'S Solution.—

Bichromate of potash . . .	2–2½ parts.
Sulphate of soda	1 part.
Water	100 parts.

The duration of the reaction is about the same as with the simple solution of chromic salt.

Recent authors find the action of this liquid to be identical with that of plain bichromate, and doubt whether the sulphate in it has any effect whatever as regards its hardening properties. Fol says that for mammalian embryos, for which it has been recommended, it is worthless.

54. **Erlicki's Solution** (*Warschauer med. Zeit.*, xxii, Nos. 15 and 18; *Progrès Médical*, 1897, No. 31).—

Bichromate of potash . .	2·5 parts.
Sulphate of copper . .	1·0 part.
Water	100·0 parts.

Here the addition of the cupric sulphate is intelligible, for this salt is itself a hardening agent of some energy. As a matter of fact, "Erlicki" hardens very much more rapidly than either simple bichromate or Müller's solution. A spinal cord may be hardened in it in four days at the temperature of an incubator, and in ten days at the normal temperature (Fol, *Lehrb. d. vergl. mik. Anat.*, p. 106). Human embryos of several months may be conveniently hardened in it.

Nerve-centres that have been hardened in Erlicki's fluid frequently contain dark spots with irregular prolongations, simulating ganglion-cells. These are now known to consist of precipitates formed by the fluid. They may be removed by washing with hot water, or with water slightly acidified with hydrochloric acid, or by treating the specimens with 0·5 per cent. chromic acid before putting them into alcohol (Tschisch, *Virchow's Arch.*, Bd. xcvii, p. 173; Edinger, *Zeit. wiss. Mik.*, ii, p. 245; Loewenthal, *Rev. méd. de la Suisse romande*, 6me année, i, p. 20).

55. **Kultschitzky's Solution** (*Zeit wiss. Mik.*, iv, 1887, p. 348).—A saturated solution of bichromate of potash and sulphate of copper in 50 per cent. alcohol, to which is added at the instant of using a little acetic acid, five or six drops per 100 c.c.

To make the solution, add the finely powdered salts to the alcohol in excess, and leave them together *in total darkness*, for twenty-four hours.

Fix for twelve to twenty-four hours *in the dark*. Then treat with strong alcohol for twelve to twenty-four hours.

56. **Dekhuyzen's Liquids** (*C. R. Acad. Sci.*, cxxxvii, 1903, pp. 415 and 445).—(A) 250 c.c. of 2·5 per cent. sol. of bichromate in sea-water, 25 c.c. of 6·3 per cent. nitric acid, and 54 c.c. of 2 per cent. osmic acid. For general use with marine animals.

(B) 173·1 c.c. of the bichromate sol. and 26·9 of 2 per cent. sol. of osmic acid. For objects containing calcareous elements that it is desired to preserve.

These liquids are stated to be isotonic with sea-water.

57. Bichromate and Sublimate (Kultschitzky, *Arch. f. mik. Anat.*, xlix, 1897, p. 8).—Two parts bichromate, $\frac{1}{4}$ part corrosive sublimate, 50 parts 2 per cent. acetic acid, and 50 parts 96 per cent. alcohol. The mixture should be filtered after twenty-four hours. Tissues of vertebrates may remain in it for four to six days. Lavdowsky (*Zeit. wiss. Mik.*, xvii, 1900, p. 301), takes 500 c.c. of 1 per cent. acetic acid, 20 to 25 g. bichromate, and 5 to 10 c.c. saturated solution of sublimate in water.

58. Bichromate of Ammonia.—This salt is in considerable favour for hardening. Its action is very similar to that of the potassium salt. Fol says that it penetrates somewhat more rapidly, and hardens somewhat more slowly. It should be employed in somewhat stronger solutions, up to 5 per cent.

59. Neutral Chromate of Ammonia is preferred by some. It is used in the same strength as the bichromate. Klein has recommended it for intestine, which it hardens, in 5 per cent. solution, in twenty-four hours.

60. Bichromate of Calcium. Sonnenbrodt (*Arch. mikr. Anat.*, lxxii, 1908, p. 416), fixes ovaries of *Gallus* in 20 parts of 2 per cent. sol. of calcium bichromate with 10 of 2 per cent. sol. of sublimate and 1 of acetic acid.

61. Bichromates and Alcohol.—Mixtures of bichromate of potash or ammonia with alcohol may be employed, and have a more rapid action than the aqueous solution. Thus Hamilton takes for hardening brain a mixture of 1 part methylated spirits with three parts of solution of Müller; see also Kultschitzky's Mixture, *ante*, § 55). Preparations should be kept in the dark during the process of hardening in these mixtures.

62. Sulphurous Acid.—Waddington (*Journ. Roy. Mic. Soc.*, 1883, p. 185) uses a saturated solution of sulphurous acid in alcohol for fixing infusoria. Overton (*Zeit. wiss. Mik.*, vii, 1890, p. 9) uses the vapours of an aqueous solution for fixing algæ.

CHAPTER V.

FIXING AND HARDENING AGENTS. CHLORIDES, ORGANIC ACIDS, AND OTHERS.

Chlorides.

63. Bichloride of Mercury (Corrosive Sublimate).—Corrosive sublimate is soluble in about sixteen parts of cold and three of boiling distilled water. It is more soluble in alcohol (1 : 3) or in ether (1 : 4) than in water. Its solubility in all these menstrua is augmented by the addition of hydrochloric acid, ammonious chloride, or camphor. With sodium chloride it forms a more easily soluble double salt; hence sea-water may dissolve over 15 per cent.

The simple aqueous solutions should always be made with *distilled*—not spring—water. The $HgCl_2$ in them is partly split up by hydrolysis into Cl, H, and $(HgCl)_2$, or HgClOH (see *Chem. Centralb.*, 1904, i, p. 571; the statements of Mann [*Methods*, pp. 22, 77] are incorrect). These solutions should give an acid reaction with litmus paper, whilst those made with strong sodium chloride solution are neutral.

For fixing, corrosive sublimate may be used pure; but in most cases a finer fixation will be obtained if it be *acidified with acetic acid*, say about 1 per cent. of the glacial acid. I find that a saturated solution in 5 per cent. glacial acetic acid is a very good formula for *marine* animals; for others I should take the acid weaker. Kaiser's solution consists of 10 g. sublimate, 3 g. glacial acetic acid, and 300 g. distilled water (from *Zeit. wiss. Mik.*, xi, p. 378). Van Beneden has used a saturated solution in 25 per cent. acetic acid, and Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) a mixture of 2 parts saturated solution with 1 part of 49 per cent. acetic acid.

It is sometimes advisable to take the most concentrated solution obtainable. For some very contractile forms (coral polypes, Planaria), a concentrated solution in *warm* or even boiling water should be employed. For Arthropoda *alcoholic* solutions are frequently indicated. Delicate objects, however, may require treatment with weak solutions.

Objects should in all cases be removed from the fixing bath *as soon as fixed,* that is, as soon as they are seen to have become opaque throughout, which may be in a few minutes or even seconds.

Wash out with water or alcohol. Alcohol is almost always preferable. Alcohol of about 70 per cent. may be taken, and (Mayer, *Intern. Monatsschr. Anat. Phys.*, iv, 1887, p. 43) a little tincture of iodine may be added to the liquid, either alcohol or water, used for washing, enough to make it of a good port-wine colour, and the mixture be changed until it no longer becomes discoloured by the objects. Apáthy (*Mikrotechnik,* p. 148) takes a 0·5 per cent. solution of iodine in strong alcohol, leaves the objects in it (suspended) until they have become of about the colour of the solution, and then washes for twenty-four hours in pure alcohol.

In *obstinate cases* solution of iodine in iodide of potassium (*e.g.* Lugol's) may be taken. Mayer (*Zeit. wiss. Mik.*, xiv, 1897, p. 28) makes it by dissolving 5 grammes of iodide of potassium in 5 c.c. of distilled water and mixing this with a solution of 0·5 gramme of iodine in 45 c.c. of 90 per cent. alcohol, but seldom uses the mixture concentrated, merely adding as much of it as is required to the alcohol or water containing the objects. The important point is, that the iodine and iodide be employed together. The iodine may be washed out in obstinate cases with magnesia water. Similarly Apáthy (*Mitth. Zool. Stat. Neapel*, xii, 1897, pp. 729, 730).

It has been objected to this process that iodine in potassic iodide precipitates corrosive sublimate instead of dissolving it. That is true, but the precipitate is soluble in excess of the precipitant.

The iodide of potassium process should be employed with care, for the iodide may partly redissolve the precipitated compounds formed by the sublimate with the albuminoids. etc., of the tissues, and it may be well not to begin adding the iodine till the objects have been brought into fairly strong alcohol, 70 or 80 per cent.

It is important that the sublimate be *thoroughly* removed from the tissues, otherwise they become *brittle,* and will not stain so well. They will also become brittle if they are kept long in alcohol.

It may happen that if the extraction of the excess of sublimate from the tissues in bulk has been insufficient, crystals may form in the sections after they have been mounted in balsam. This may easily be prevented by treating the sections themselves with tincture of iodine for a quarter of an hour before mounting. Some workers hold that this does away with the necessity of treating the tissues in bulk with iodine, which is frequently a very long process. Thus, Mann (*Zeit. wiss. Mik.*, xi, 1894, p. 479) prefers treating the sections rather than the tissues in bulk, on the ground that the iodine makes them soft, so that they shrink on coming into paraffin. Schaper (*Anat. Anz.*, xiii, 1897, p. 463), however, has shown that neglect to extract the sublimate from the tissues in bulk may give birth to serious artefacts, which appear to arise during the imbedding process. So also Loyez (*Arch. Anat. Micr.*, viii, 1905, p. 71). Heidenhain (*Zeit. wiss. Mik.*, xxv, 1909, p. 398) removes the iodine from sections by means of sodium thiosulphate.

You may stain in any way you like. Carmine stains are peculiarly brilliant after sublimate.

The solutions must not be touched with iron or steel, as these produce precipitates that may hurt the preparations. To manipulate the objects, wood, glass or platinum may be used; for dissecting them, hedgehog spines, or quill pens, or cactus spines.

When properly employed, sublimate is *for general work* undoubtedly a most useful fixing agent. It is applicable to most classes of objects. It is perhaps less applicable, in the pure form, to Arthropods, as it possesses no great power of penetrating chitin. For *cytological work* it is, according to my experience, not to be trusted, and only to be recommended where more precise fixing agents are counter-indicated by reason of their lack of penetration, or the like. Amongst other defects it has that of frequently causing *very serious shrinkage* of cells.

64. Sublimate with Salt.—A solution containing 5 g. sublimate, 0·5 g. sodium chloride, and 100 c.c. water has been quoted as "solution of Gaule."

A one-half per cent. aqueous solution of sodium chloride saturated whilst hot with sublimate was much recommended by Heidenhain (*Festschrift f. Koelliker*, 1892, p. 109).

The addition of sodium chloride allows a stronger solution to be obtained than can be made with pure water, and also, it is stated, enhances the penetration of the sublimate. But the fixation-precipitates (§ 29) formed by the double salt are (according to Spuler, *Encycl. mik. Technik.*, p. 1274) for the most part soluble in water, thus giving rise to imperfect preservation.

Concentrated (i. e. over 20 per cent.) solution in sea-water is recommended for some marine animals.

Stoelzner (*Zeit. wiss. Mikr.*, xxiii, 1906, p. 25) recommends saturated solution of sublimate in sugar solution of 4½ per cent., as isotonic (for warm-blooded animals).

Liquid of Lang (*Zool. Anzeiger*, 1878, i, p. 14).—For *Planaria.*—

Distilled water	100.
Chloride of sodium . . .	6 to 10.
Acetic acid	6 to 8.
Bichloride of mercury . .	3 to 12.
(Alum, in some cases . .	½.)

65. Alcoholic Solutions.—Apáthy (*Mikrotechnik*, p. 111) recommends a solution of 3 to 4 grammes of sublimate and 0·5 gramme sodium chloride in 100 c.c. of 50 per cent. alcohol, for *general* purposes.

Ohlmacher (*Journ. Exper. Medicine*, ii, 6, 1897, p. 671) takes—

Absolute alcohol	80 parts.
Chloroform	15 ,,
Glacial acetic acid	5 ,,

Sublimate to saturation (about 20 per cent.).

"Ordinary pieces" of tissue are sufficiently fixed in fifteen to thirty minutes. Entire human cerebral hemispheres, subdivided by Meynert's section, take eighteen to twenty-four hours.

For liquids containing a much higher proportion of acetic acid, see *Acetic Alcohol.*

66. Aceton Solution.—Held (*Arch. Anat. Phys.*, Anat. Abth., 1897, p. 227), fixes nerve-tissue in a 1 per cent. solution of sublimate in 40 per cent. aceton, and washes out through increasingly concentrated grades of aceton.

67. Phenol Solution.—Pappenheim (*Arch. Path. Anat.*, clvii, 1899, p. 23) shakes up carbolic acid with aqueous sublimate solution and filters.

68. Ciaccio (*Arch. Ital. Anat. Embr.*, vi, 1907, p. 486) has an irrational mixture of sublimate, iodine, and formol.

69. Mercuro-nitric Mixtures.—Frenzel (*Arch. mik. Anat.*, xxvi, 1885, p. 232) recommends a half-saturated solution of sublimate in 80 per cent. alcohol, to which is added nitric acid in the proportion of 1 drop to 1 c.c. or 2 c.c. Objects of the size of a pea to be fixed in it for five or ten minutes, then hardened in the same sublimate alcohol without the acid, and finally in 90 per cent. alcohol. It is said that the nitric acid renders after-treatment with iodine unnecessary.

Gilson's **Mixture** (Gilson, *in litt.* 1895).

Nitric acid of 46° strength (this would be sp. gr. 1·456, or 80 per cent., nearly)	15 c.c.
Glacial acetic acid	4 ,,
Corrosive sublimate	20 grm.
60 per cent. alcohol	100 c.c.
Distilled water	880 ,,

When required *for marine animals* add a few crystals of iodine, which will prevent the formation of precipitates of sea salts. If in any case the preparations should show a granular precipitate, this may be removed by washing with water containing a little tincture of iodine.

I find that it affords in general a faithful and delicate fixation, and gives to tissues an excellent consistency. Objects may remain in it for a considerable time without hurt. It has a high degree of penetration. A treatment for a few days with it will serve to remove the albumen from the ova of Batrachians. This liquid *may be recommended to beginners*, as it is very easy to work with. For some objects, as I found, the proportion of sublimate may be increased with advantage.

Kostanecki and Siedlecki (*Arch. mik. Anat.*, xlviii, 1896, p. 181) take a mixture of saturated sublimate solution and 3 per cent. nitric acid in equal parts, or a mixture of equal parts of sublimate solution, 3 per cent. nitric acid, and absolute alcohol, fix for twenty-four hours, and wash out in iodine-alcohol.

Petrunkewitsch (*Zool. Jahrb. Abth. Morph.*, xiv, 1901, p. 576) takes water 300, absolute alcohol 200, glacial acetic acid 90, nitric acid 10, and sublimate to saturation. Both this and Gilson's have been much used lately.

70. Picro-sublimate Mixtures.—Rabl's (*Zeit wiss. Mik.*, xi, 1894, p. 165). Sublimate, saturated solution in water, 1 vol.; a similar solution of picric acid, 1 vol.; distilled water 2 vols. Embryos may be left in it for twelve hours washed for two hours in water, and brought into weak alcohol.

Mann's (*op. cit.*, xi, 1895, p. 480).—1 per cent. of picric acid with or without 1 per cent. of tannin in a saturated solution of sublimate in normal salt solution.

The same author's **Alcoholic Picro-sublimate** (*Anat. Anz.*, viii, 1893, pp. 441—443) consists of absolute alcohol 100 c.c., picric acid 4 grms., sublimate 15 grms., tannin 6 to 8 grms. The tannin is added in order to prevent excessive hardening.

Pacaut (*Arch. Anat. Micr.*, viii, 1906, p. 438), takes 200 parts of saturated solution of sublimate and picric acid, 6 of 3 per cent. platinum chloride, and 5 of 16½ per cent. solution of chromic acid.

O. vom Rath (*Anat. Anz.*, xi, 1895, p. 268) takes cold saturated solution of picric acid, 1 part; hot saturated solution of sublimate, 1 part; glacial acetic acid, ½ to 1 per cent. Also the same with the addition of 10 per cent. of 2 per cent. osmic acid solution.

Fish (*Trans. Amer. Micr. Soc.*, xvii, 1896, p. 143) takes 1 litre of water, 1 g. picric acid, 5 g. sublimate, and 10 g. acetic acid.

Lenhossek (*Intern. Monatschr. Anat. Phys.*, xxiv, 1907, p. 293) takes saturated sublimate 75 c.c., acetic acid 5, 50 per cent. alcohol 25, and picric acid to saturation.

71. Osmio-sublimate Mixtures.—Mann's (*Zeit. wiss. Mik.*, xi, 1894, p. 481) consists of a freshly prepared mixture of equal parts of 1 per cent. osmic acid solution and saturated solution of sublimate in normal salt solution (for nerve-centres).

Drüner's (*Jena. Zeit. Naturw.*, xxviii, 1894, p. 294) consists of 1 part of 1 per cent. osmic acid solution added to 20 parts of a solution of 5 per cent. each of sublimate and glacial acetic acid in water.

O. vom Rath's, see last §.

72. Chromo-sublimate.— Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 3, 1890, p. 443). Concentrated sublimate solution, 100 parts, 1 per cent. chromic acid, 50 parts.

Mann (*Verh. Anat. Ges.*, 12, 1898, p. 39) takes for nerve-cells equal parts of 5 per cent. sublimate and 5 per cent. chromic acid.

73. Sublimate and Bichromate.—Zenker's **Mixture** (*Münchener* *med. Wochenschr.*, 24, 1894, p. 534; quoted from MERCIER, *Zeit. wiss. Mik.*, xi, 4, 1894, p. 471). Five per cent. of sublimate and 5 per cent. of glacial acetic acid dissolved in solution of MÜLLER. Fix for several hours, wash out with water, treat the tissues in bulk, or the sections with alcohol containing tincture of iodine.

See also RETTERER, *Journ. Anat. Phys.*, xxxiii, 1897, p. 463, and xxxvii, 1901, p. 480.

If the objects be allowed to remain too long in the fluid there may be formed precipitates, which it is very difficult to remove. SPULER (*Encycl. mik. Technik.*, 1st edition, p. 1280) says that they may be avoided by removing the objects as soon as penetrated, and completing the hardening in liquid of MÜLLER.

DAHLGREN'S modification, consisting of equal parts of Müller's solution and saturated sublimate solution with 5 per cent. of glacial acetic acid, gives fewer precipitates (SPULER, *l. c.*).

HELLY (*Zeit. wiss. Mik.*, xx, 1904, p. 413) omits the acetic acid, and adds, immediately before use, 5 per cent. of formol.

MAXIMOW (*ib.*, xxvi, 1909, p. 179) adds 10 per cent. of formol and sometimes 10 per cent. of osmic acid of 2 per cent. (fix in the dark).

FOÀ (*Quart. Journ. Mic. Sci.*, 1895, p. 287) takes equal parts of saturated solution of sublimate in normal salt solution, and of liquid of Müller, or 5 per cent. solution of bichromate.

BENSLEY (*Proc. Canadian Inst.*, v, 1897, p. 77; *Zeit. wiss. Mik.*, xvii, 1900, p. 233) takes equal parts of saturated solution of sublimate in 96 per cent. alcohol and 2 per cent. solution of bichromate in water. Wash out in 50 per cent. alcohol.

HOYER (*Arch. Mikr. Anat.*, liv, 1899, p. 97) takes 1 part 5 per cent. sublimate and 2 of 3 per cent. bichromate.

KOHN (*ib.*, lxx., 1907, p. 273) takes 5 parts 5 per cent. sublimate, 15 parts 3½ per cent. bichromate, and 1 part acetic acid.

ARNOLD (*Arch. Zellforsch.*, iii, 1909, p. 433) takes 2·5 parts bichromate, 1 of cupric sulphate, 10 of acetic acid, and 100 of saturated solution of sublimate.

74. Sublamin (Ethylendiamin Sulphate of Mercury) is recommended in 5 per cent. solution by KLINGMÜLLER and VEIEL, *Zeit. wiss. Mikr.*, xxi, 1904, p. 58.

75. Platinum Chloride.—The substance used and intended by the authors who have recommended this reagent is not the true platinic chloride, or tetrachloride, $PtCl_4$, but the compound H_2PtCl_6, that is, platinochloric, or hydro-chloroplatinic acid, by custom called platinum chloride. It occurs as brown-red crystals, easily soluble in water and very deliquescent. For this reason it had better be stocked in the form of a 10 per cent. solution, kept in the dark (weak solutions—0·5 per cent.—may be kept in the light).

It appears that some authors have stated that they were using platinous chloride, $PtCl_2$, but that is not possible, as this salt is not soluble in water.

Rabl (*Morph. Jahrb.*, x, 1884, p. 216) employed an aqueous solution of 1·300. The objects remained in it for 24 hours, and were then washed out with water. Well-washed preparations give good chromatin stains with the "basic" tar colours; but I find, as do others, that plasma-staining with the "acid" colours is rendered extremely difficult. It causes a certain shrinkage of chromatin.

It is now almost always employed in the form of mixtures. For these see §§ 44, 45, 49, 76, as well as the mixtures given under Picric acid and Formol.

76. Rabl (*Zeit. wiss. Mikr.*, xi, 1894, p. 165) takes for embryos of vertebrates, and also for other objects, 1 vol. of 1 per cent. platinum chloride, 1 of saturated sublimate, and 2 of water.

Lenhossek (*Arch. Mikr. Anat.*, li, 1898, p. 220) takes 20 parts of 1 per cent. platinum chloride, 20 of 5 per cent. sublimate, and 1 of acetic acid.

77. **Palladium Chloride** (Schulze, *Arch. mik. Anat.*, iii, 1867, p. 477).—Used by Schulze as a hardening agent in a 1 : 800 solution, acidified with hydrochloric acid.

Cattaneo has used it in solutions of 1·300, 1·600, or 1·800 strength, for from one to two minutes, for Infusoria.

Frenkel (*Anat. Anz.*, viii, 1893, p. 538) recommends for connective tissue a mixture of 15 parts 1 per cent. palladium chloride, 5 parts 2 per cent. osmic acid, and a few drops of acetic acid.

78. **Iridium Chloride** (Eisen, *Zeit. wiss. Mik.*, xiv, 1897, p. 195).—Solution of one half or one fifth per cent., acidified with 1 per cent. of glacial acetic acid.

With the ovotestis of the snail, I have obtained about the worst fixation I have ever seen, but with the testis of *Triton* much better results.

79. **Osmium Chloride** (Eisen, *Journ. of Morph.*, xvii, 1900).—Solution

of $\frac{1}{2}$ to $\frac{1}{10}$ per cent. From specimens I have seen I should say it is useless.

80. **Perchloride of Iron** (Fol, *Zeit. wiss. Zool.*, xxxviii, 1883, p. 491, and *Lehrb. d. vergl. mik. Anat.*, p. 102).—Fol recommends 1 vol. of *Tinct. Ferri Perchlor.* B.P. diluted with 5 to 10 vols. of 70 per cent. alcohol.

The tincture diluted with 3 to 4 vols. of either alcohol or water has been recommended for fixing medullated nerve by Platner (*Zeit. wiss. Mik.*, vi, 1889, p. 187).

81. **Iron Alum.**—Strong (*Journ. comp. Neur.*, xiii, 1903, p. 296) fixes (and decalcifies) heads of young *Acanthias* in 9 parts of 5 per cent. solution of iron alum with 1 of formol, for about two weeks.

82. **Chloride of Zinc** is sometimes used for hardening brain (see Part II). Gilson (*La Cellule*, vi, 1890, p. 122) has used it as a fixative for the silk glands of Lepidoptera, as follows :

Glacial acetic acid	5	c.c.
Nitric acid of 46° (or 80 per cent. nearly) .	5	,,
Alcohol of 80 per cent.	100	,,
Distilled water	300	,,
Dry chloride of zinc	20	grms.

83. **Iodine.**—Kent (*Manual of the Infusoria*, 1881, p. 114) uses it for fixing Infusoria. Prepare a saturated solution of potassic iodide in distilled water, saturate this solution with iodine, filter, and dilute to a brown-sherry colour. A very small portion only of the fluid is to be added to that containing the Infusoria.

Or you may use Lugol's solution :

Water	100	parts.
Iodide of potassium	6	,,
Iodine	4	,,

Or for small marine animals, a solution of iodine in sea-water.

Personally I have found it very useful for the examination of *spermatozoa*.

Very small objects may be instantaneously fixed by means of **vapour of Iodine.** Crystals of iodine may be heated in a test-tube till the vapours are given off ; then on inclining the tube the heavy vapours may be made to flow over the objects arranged on a slide. The slide should then be warmed to about 40° C. for one to three minutes in order to evaporate the iodine from the objects, which may then be mounted or otherwise treated as desired (Overton, *Zeit. wiss. Mik.*, vii, 1890, p. 14).

Organic Acids, and other Agents.

84. **Acetic Acid.**—Flemming, who has made a special investigation of its action on *nuclei*, finds (*Zellsubstanz*, etc., p. 380) that the best strength is from 0·2 to 1 per cent.

Strengths of 5 per cent. and more bring out the nuclein structures clearly at first, but after a time cause them to swell and become pale, which is not the case with the weaker strengths (*ibid.*, p. 103). The *strong* acid is, however, a valuable fixative of certain objects, which it kills with the utmost rapidity, *and leaves fixed in a state of extension.* The *modus operandi* of VAN BENEDEN is as follows:—Pour glacial acetic acid in liberal quantity over the organisms, leave them until they are penetrated by it—which should be in five or six minutes, as the strong acid is a highly penetrating reagent—and wash out in frequent changes of alcohol of gradually increasing strength. Some persons begin with 30 per cent. alcohol, but this appears to me rather weak, and I think 70 per cent. or at least 50 per cent. should be preferred.

Other energetic reagents may be combined with the glacial acetic acid if desired. Dr. LINDSAY JOHNSON (*in. litt.*) has found that one of the best fixatives for retina is a mixture of equal parts glacial acetic acid and 2 per cent. osmic acid. S. LO BIANCO adds to the "concentrated"* acid one tenth of a 1 per cent. solution of chromic acid. He finds that even this small proportion of chromic acid serves to counteract in a marked degree the softening action of the acetic acid.

Acetic acid, used *alone*, is only a fixative for *a limited time.* If its action be prolonged, it becomes a *swelling* agent. Its function in mixtures is, besides that of *killing*, the valuable one of counteracting the shrinking action of the ingredients with which it is combined, and by its swelling action enhancing the *penetration* of the mixture; whilst by clarifying tissues it adds in the optical differentiation of their elements.

The proportions in which it should enter into mixtures in general seem to me to be from 0·5 per cent. to 5 per cent. of the glacial acid; higher strengths, such as 25 per cent. to 100 per cent., being only indicated in cases in which the highest possible penetration is the chief consideration.

Throughout this work, wherever acetic acid is mentioned, it is the *glacial* acid that is meant unless the contrary is stated.

---

* The acid referred to as "concentrated" by LO BIANCO in his *Metodi* (*Mitth. Zool. Stat. Neapel*, xi, 3, p. 435) is an acid of approximately 49 per cent. (sp. gr. 1·060).

All liquids containing a large proportion of this acid (*e. g.* §§ 85, 86) should only be allowed to act for a *very short time.*

85. Acetic Alcohol (Carnoy, *La Cellule*, iii, 1886, p. 6; and *ibid.*, 1887, p. 276; v. Beneden et Neyt, *Bull. Ac. Sci. Belg.*, xiv, 1887, p. 218; Zacharias, *Anat. Anz.*, iii, 1888, pp. 24—27; v. Gehuchten, *ibid.*, 8, p. 227).—Carnoy has given two formulæ for this important reagent. The first is—

Glacial acetic acid . . .	1 part.
Absolute alcohol . . .	3 parts.

The second is—

Glacial acetic acid . . .	1 part.
Absolute alcohol . . .	6 parts.
Chloroform	3 ,,

The addition of chloroform is said to render the action of the mixture more rapid.

V. Beneden and Neyt take equal volumes of glacial acid and absolute alcohol.

Zacharias takes—

Glacial acetic acid . . .	1 part.
Absolute alcohol . . .	4 parts.
Osmic acid	a few drops.

Acetic alcohol is one of the most penetrating and quickly acting fixatives known. It preserves both nuclei and cytoplasm, and admits of staining in any way that may be preferred. It was employed by all of the authors quoted for the ova of *Ascaris*—proverbially one of the most difficult objects to fix,—but I have found that it is applicable to many other objects. Wash out with alcohol, and avoid aqueous liquids as far as possible in the after-treatment.

86. Acetic Alcohol with Sublimate.—Carnoy and Lebrun (*La Cellule*, xiii, 1, 1887, p. 68, due to Gilson).

Absolute alcohol	1 vol.
Glacial acetic acid	1 ,,
Chloroform	1 ,,

Sublimate to saturation.

(The mixture does not keep long, forming ethyl acetate, which precipitates.)

Isolated ova of *Ascaris,* even though furnished with a shell, are fixed in twenty-five to thirty seconds. Entire oviducts take about ten minutes. The liquid is therefore one of the most penetrating and rapidly acting of any, if not *the* most.

Wash out with alcohol until all traces of odour of the acetic acid have disappeared (I myself wash out with alcohol containing tincture of iodine). I consider this a very fine reagent.

For Ohlmacher's mixture see § 65.

MINGAZZINI'S MIXTURE (*Ricerche Lab. Anat. Roma,* iii, 1893, p. 47).—Two vols. saturated aqueous solution of sublimate, one of absolute alcohol, and one of glacial acetic acid.

87. Trichlor-acetic Acid (HOLMGREN, *Anat. Hefte,* xviii, 1901, H. 2).—5 per cent. solution in water. Fix (nerve-cells) for 8 to 24 hours, wash out with alcohol. See also HEIDENHAIN, *Zeit. wiss. Mikr.,* xxii, 1905, p. 321, and xxv, 1909, p. 405, who makes a mixture of 6 per cent. sublimate solution with 2 per cent. of trichlor-acetic and 1 per cent. of acetic acid, which he calls "Subtriessig."

88. Trichlor-lactic Acid (HOLMGREN, *Anat. Anz.,* xx, 1902, p. 435).—As the last. Gives rise to serious swelling.

89. Salicylic Acid (HEIDENHAIN, *Arch. mik. Anat.,* liv, 1899, p. 186).—Saturated solution in one-third alcohol. A trial has given me simply atrocious results.

90. Chloride and Acetate of Copper (*Ripart et Petit's Liquid,* CARNOY, *La Biologie Cellulaire,* p. 94).—

Camphor water (not saturated)	75 grms.
Distilled water	75 ,,
Crystallised acetic acid	1 grm.
Acetate of copper	0·30 ,,
Chloride of copper	0·30 ,,

This is a very moderate and delicate fixative, extremely useful for objects that are to be studied in *as fresh a state as possible* in aqueous media. Objects fixed in it stain instantaneously and perfectly with methyl green. Osmic acid may be added to the liquid to increase the fixing action. For *cytological researches* a valuable medium.

91. **Nitrate of Copper** (GILSON, from GELDERD, *La Cellule*, xxv, 1909, p. 12).—Nitrate of copper 200, formol 500, sea-water 200. Seven parts of this solution to be diluted with 100 of sea-water. For Crustacea.

92. **Acetate of Uranium** (SCHENK, *Mitth. Embryol. Inst. Wien*, 1882, p. 95; cf. GILSON, *La Cellule*, i, 1885, p. 141) has a mild fixing action, and a high degree of penetration, and may be combined with methyl green.

FRIEDENTHAL (*Sitzb. Ges. Nat. Freunde Berlin*, 1907, p. 209) recommends equal parts of saturated solution of the acetate and trichloracetic acid of 50 per cent.

93. **Picric Acid.**—Picric acid in aqueous solution should be employed in the form of a *strong* solution whenever it is desired to make sections or other preparations of tissues with the elements *in situ*, as weak solutions macerate; but for dissociation preparations or the fixation of isolated cells, weak solutions may be taken. Flemming finds that the fixation of nuclear figures is equally good with strong or weak solutions. The saturated solution is the one most employed. (One part of picric acid dissolves in about 86 parts of water at 15° C.; in hot water it is very much more soluble.) Objects should remain in it for from a few seconds to twenty-four hours, according to their size. For Infusoria one to at most two minutes will suffice, whilst objects of a thickness of several millimetres require several hours.

Picric acid should *always be washed out with alcohol*, that of 70 per cent. being mostly indicated. Staining should be performed by means of alcoholic solutions, or if with aqueous, then with such as are themselves weak hardening agents, such as hæmalum, carmalum, methyl green.

Washing out is facilitated by heat, the extraction being about twice as rapid at 40° C. as at the normal temperature (FOL).

It has been found by JELINEK (*Zeit. wiss. Mik.*, xi, 1894, p. 242) that the extraction is greatly quickened by the addition of a base to the wash-alcohol. He recommends carbonate of lithia. A few drops of a saturated solution of the salt in water are added to the alcohol; a precipitate is formed. The objects are put into the turbid alcohol, which becomes clear and yellow in proportion as the picrin is extracted. Further quantities of carbonate are added from time to time until the colour has been entirely extracted.

Tissues fixed in picric acid can be perfectly stained in any stain. It is seldom necessary to remove the picric acid by washing out before staining. Paracarmine, Borax-carmine, or Hæmacalcium may be recommended for entire objects.

The most important property of picric acid is its great penetration. This renders it peculiarly suitable for the preparation of chitinous structures.

94. Picric Alcohol (Gage, *Proc. Amer. Soc. Micr.*, 1890, p. 120).—Alcohol (95 per cent.), 250 parts; water, 250 parts; picric acid, 1 part.

95. Picro-acetic Acid.—Boveri (*Zellenstudien*, 1, 1887, p. 11) dilutes a concentrated aqueous solution of picric acid with two volumes of water and adds 1 per cent. of acetic acid. According to my experience, the results are miserable.

Zimmer's mixture (from Deegener, *Zool. Jahrb. Abth. Morph.*, xxvii, 1909, p. 634).—Saturated aqueous solution of picric acid, 10 parts; absolute alcohol, 9; acetic acid, 1.

96. Picro-sulphuric Acid (Kleinenberg, *Quart. Journ. Mic. Sci.*, April, 1879, p. 208; Mayer, *Mitt. Zool. Stat. Neapel*, ii, 1880, p. 2).—Mayer takes distilled water, 100 vols.; sulphuric acid, 2 vols.; picric acid, as much as will dissolve.

Liquid of Kleinenberg is made by diluting the concentrated picro-sulphuric acid prepared as above with three times its volume of water.

I hold that the concentrated solution is generally preferable. *This particularly applies to marine organisms.*

Wash out with successive alcohols, beginning with 70 per cent., never with water.

Warm alcohol extracts the acid much more quickly than cold, without which *weeks* may be required to fully remove the acid from chitinous structures.

This liquid may still be useful for Arthropoda, on account of its great power of penetrating chitin; and for some embryological purposes. For a fuller account *see early editions.*

97. Picro nitric acid (Mayer, *Mitth. Zool. Stat. Neapel*, 1881 p. 5).—

Water	100 vols.
Nitric acid (of 25 per cent. N_2O_5) . .	5 ,,

Picric acid, as much as will dissolve.

Properties of this fluid similar to those of picro-sulphuric acid, with the advantage of avoiding the formation of gypsum crystals, and the disadvantage that it is much more difficult to soak out of the tissues. Mayer states that with eggs containing a large amount of yolk material, like those of Palinurus, it gives better results than nitric, picric, or picro-sulphuric acid. I myself consider it distinctly superior to picro-sulphuric for most things.

98. Picro-hydrochloric Acid (Mayer, *ibid.*).—

Water	100 vols.
Hydrochloric acid (of 25 per cent. HCl) .	8 ,,

Picric acid, as much as will dissolve.

99. Picro-chromic Acid (Fol, *Lehrb.*, p. 100).—

Picric acid, sol. sat. in water . .	10 vols.
1 per cent. chromic acid solution . .	25 ,,
Water	65 ,,

I have seen Fol's formula, with the addition of a trace of acetic acid, quoted as "liquid of Haensel."

Lo Bianco takes equal parts of picro-sulphuric acid and chromic acid of 1 per cent.

Rawitz (*Leitfaden*, 1895, p. 24) takes 1 part of picro-nitric acid, and four parts 1 per cent. chromic acid. Wash out in 70 per cent. alcohol.

100. Picro-osmic Acid.—Flemming (*Zells. Kern u. Zellth.*, p. 381) has experimented with mixtures made by substituting picric for chromic acid in the chromo-osmic mixtures (§ 42), and finds the results identical, so far as regards the fixation of nuclei. The fixation of cytoplasm is in my preparations decidedly inferior.

O. vom Rath (*Anat. Anz.*, xi, 1895, p. 289) adds to 200 c.c. of saturated aqueous solution of picric acid, 12 c.c. of 2 per cent. solution of osmic acid, and 2 c.c. of glacial acetic acid.

Rawitz (*Leitfaden*, p. 24) takes picro-nitric acid, 6 vols.; 2 per cent. osmic acid, 1 vol. Fix for ½ to 3 hours. Transfer direct to 70 per cent. alcohol.

101. Picro-platinic and Picro-platin-osmic Mixtures.—O. vom Rath (*loc. cit.*, last §, pp. 282, 285) makes a picro-platinic mixture with 200 c.c. saturated aqueous solution of picric acid, 1 g. of platinic chloride (dissolved in 10 c.c. of water), and 2 c.c. of glacial acetic acid.

The picro-platin-osmic mixture, which is, in my opinion, much superior, is made by adding to the foregoing 25 c.c. of 2 per cent. osmic acid.

Other Picric Mixtures. See §§ 70 and 110 to 112.

Other Fixing and Hardening Agents.

102. Alcohol.—For *fixing* only two grades of alcohol should be employed—very weak, or absolute. Absolute alcohol ranks as a fixing agent because it kills and hardens with such rapidity that structures have hardly time to get deformed in the process; very weak, because it possesses a sufficiently energetic coagulating action and yet contains enough water to have but a feeble dehydrating action. The intermediate grades do not realise these conditions, and therefore should not be employed *alone* for fixing. But they may be very useful in combination with other fixing agents by enhancing their penetrating power; 70 per cent. is a good grade for this purpose.

Table for diluting alcohol (after GAY-LUSSAC).—To use this table, find in the upper horizontal row of figures the percentage of the alcohol that it is desired to dilute, and in the vertical row to the left the percentage of the alcohol it is desired to arrive at. Then follow out the vertical and horizontal rows headed respectively by these figures, and the figure printed at the point of intersection of the two rows will show how many volumes of water must be taken to reduce *one hundred volumes* of the original alcohol to the required grade.

Weaker grade required.	ORIGINAL GRADE.								
	90 p. 100.	85 p. 100.	80 p. 100.	75 p. 100.	70 p. 100.	65 p. 100.	60 p. 100.	55 p. 100.	50 p. 100.
p. 100. 85	6·56								
80	13·79	6·83							
75	21·89	14·48	7·20						
70	31·05	23·14	15·35	7 64					
65	41·53	33·03	24·66	16·37	8·15				
60	53·65	44·48	35·44	26·47	17·58	8·76			
55	67·87	57·90	48 07	38·32	28·63	19·02	9·47		
50	84·71	73·90	63·04	52·43	41·73	31·25	20·47	10·35	
45	105·34	93·30	81·38	69·54	57 78	46·09	34·46	22 90	11·41
40	130·80	117·34	104·01	90·76	77·58	64·48	51·43	38·46	25·55
35	163·28	148·01	132 88	117·82	102·84	87·93	73·08	58·31	43·59
30	206 22	188·57	171·05	153·61	136·04	118.94	101·71	84·54	67·45

Alcohol is an easily *oxidisable* substance. Chromic acid, for instance, easily oxidises it, first into aldehyde, and then into acetic acid. It follows that alcohol should not be combined in mixtures with oxidising agents of notable energy. Further, alcohol is a *reducing* agent, and therefore should not be combined with easily reducible substances. These remarks particularly apply to chromic acid, see §§ 37, 38, 48.

For *fixing,* alcohol is a very third-class reagent, only to be used *alone* where better ones cannot be conveniently employed, though it enters as a useful ingredient into many *mixtures,* in which it serves to enhance the power of penetration. For *hardening* it is an important one. 90 to 95 per cent. is the most generally useful strength. Weaker alcohol, down to 70 per cent., is often indicated. Absolute alcohol is seldom advisable. You ought to begin with weak, and proceed gradually to stronger, alcohol. Large quantities of alcohol should be taken. The alcohol should be frequently changed, or the tissue should be suspended near the top of it (§ 31). Many weeks may be necessary for hardening large specimens. Small pieces of permeable tissue, such as mucous membrane, may be sufficiently hardened in twenty-four hours.

103. Absolute Alcohol.—This is sometimes valuable on account of its great penetrating power. Mayer finds that boiling absolute alcohol is often the only means of killing certain Arthropoda rapidly enough to avoid maceration.

It is important to employ for fixing a very large proportion of alcohol. Alum-carmine is a good stain for small specimens so fixed. For preservation, the object should be put into a weaker alcohol, 90 per cent. or less.

As to the supposed superiority of absolute alcohol over ordinary strong alcohol, see last § ; and amongst authors upholding its superiority, see besides RANVIER, MAYER (*Mitth. Zool. Stat. Neapel,* ii, 1880, p. 7) ; BRÜEL (*Zool. Jahrb., Abth. Morph.,* x, 1897, p. 569) ; and VAN REES (*ibid.,* iii, 1888, p. 10).

Absolute alcohol is a product that it is almost impossible to preserve in use, on account of the rapidity with which it hydrates on exposure to air. Fol recommends that a little quicklime be kept in it. This absorbs part at least of the moisture drawn by it from the air.

Another plan that I have seen recommended is to suspend strips of gelatin in it. But it is probably rendered very acid thereby.

Ranvier prepares a sufficiently "absolute" alcohol as follows:—Strong (95 per cent.) alcohol is treated with calcined cupric sulphate, with which it is shaken up and allowed to remain for a day or two. It is then decanted and treated with fresh cupric sulphate, and the operation is repeated until the fresh cupric sulphate no longer becomes conspicuously blue on contact with the alcohol; or until, on a drop of the alcohol being mixed with a drop of turpentine, no particles of water can be seen in it under the microscope. The cupric sulphate is prepared by calcining common blue vitriol in a porcelain capsule over a flame until it becomes white, and then reducing it to powder (see *Proc. Acad. Nat. Sci. Philad.*, 1884, p. 27; *Journ. Roy. Mic. Soc.*, 1884, pp. 322 and 984).

Test for the presence of water (YVON, *C. R. Acad. Sci.*, 1897, p. 1181).—Add coarsely powdered calcium carbide; the merest trace of water will cause an evolution of acetylene gas, and on agitation the alcohol will become turbid.

104. One-third Alcohol.—The grade of weak alcohol that is generally held to be most useful for fixing is one-third alcohol, or RANVIER'S ALCOHOL. It consists *of two parts of water and one part of alcohol of* 90 *per cent.* (and *not* of absolute alcohol). See the *Traité Technique* of Ranvier, p. 241, *et passim.*

Objects may be left for twenty-four hours in this alcohol; not more, unless there be no reason for avoiding *maceration*, which will generally occur after that time. You may conveniently stain with picro-carmine, alum-carmine, or methyl green.

This reagent is a very mild fixative. Its hardening action is so slight that it is not at all indicated for the fixing of objects that are intended to be sectioned. Its chief use is for extemporaneous and dissociation preparations.

105. Acid Alcohol (MAYER, *Mitth. Zool. Stat. Neapel*, ii, 1881, p. 7).—To 97 vols. of 90 per cent. alcohol add 3 vols. pure hydrochloric (or nitric) acid. Wash out with 90 per cent. alcohol.

The use of this mixture is principally for the *preparation of museum specimens.*

LO BIANCO (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) takes 50 per cent. alcohol with 5 per cent. of hydrochloric acid.

106. Pyridin.—Pyridin has been recommended as a hardening agent (by A. DE SOUZA). It hardens, dehydrates, and clears at the same time. It is said to harden quickly, and to give particularly good results with brain. See *Comptes Rendus hebd. de la Soc. de Biologie*, 8 sér., t. iv, 1887, p. 622.

This substance is strongly alkaline, and, either pure or diluted with water, dissolves many albumens and fats. It causes considerable shrinkage of nuclei (not so much of cytoplasm). It is now in much use in certain neuro-fibril stains, see BIELSCHOWSKY and RAMÓN. It is soluble in water and in alcohol. Pure, it will harden and dehydrate small brains in a week.

107. ACETONE is said to harden very rapidly. SCHOLZ (*Zeit. wiss. Mikr.*, xxii, 1905, p. 415) fixes small objects in warm acetone for half an hour to an hour and brings them direct, or through alcohol and ether, into celloidin.

Similarly FUSS (*Arch. path. Anat.*, clxxxv, 1906, p. 5), using it cold, and LINTWAREW (*ibid.*, ccvi, 1911, p. 36) for erythrocytes, in which it preserves the hæmoglobin.

108. Formaldehyde, Formic Aldehyde, Methyl Aldehyde (Formol, Formalin, Formalose).—Formaldehyde is the chemical name of the gaseous compound HCOH, obtained by the oxidation of methyl-alcohol. "Formol," "Formalin," and "Formalose" are commercial names for the saturated (40 per cent.) solution of this in distilled water. This quickly loses in strength through contact with air, and laboratory solutions rarely contain more than 38 per cent. of formaldehyde.

Much confusion has been caused by indiscriminate use of the terms "formaldehyde" and "formol." The proper way is evidently either to state the strengths of solutions in terms of formaldehyde, and say so; or to say "formol—or formalin—with so many volumes of water." The majority of writers seem to state in terms of *formol*.

Solutions of formaldehyde sometimes decompose partially or entirely, with formation of a white deposit of paraformaldehyde. FISH says that to avoid this the solution should be kept in darkened bottles in the cool, or, according to some, it suffices to add glycerin to them.

The solutions almost always have an acid reaction, due to the presence of formic acid; but that is, as a rule, rather an advantage. But some observers hold that neutral or feebly alkaline solutions fix better than acid ones. Solutions may be neutralised by the usual methods: it will generally suffice to make them up with spring water.

It was said above that formaldehyde possesses *certain* hardening and preserving qualities. It hardens gelatine, for instance, and certain albuminoids; but others, on the contrary, are not hardened by it, but sometimes even rendered more soluble than they are naturally. For some theoretical considerations concerning its action on tissues, see F. BLUM, in *Anat. Anz.*, xi, 1896, p. 718; BENEDECENTI, in *Arch. Anat. u. Phys.*, Abth., 1897, p. 219; GEROTA, in *Intern. Monatschr. Anat.*, xiii, 1896, p. 108; *Zeit. wiss. Mik.*, xiii, p. 311; SJÖBRING in *Anat. Anz.*, xvii, 1900, p. 274; and BLUM, in *Encycl. Mik. Technik.*, p. 393. It seems to be generally admitted that this action consists in the formation of methylene compounds with the substances of the tissues.

I find that, used *pure*, it is far from a first-class fixative. For it over-fixes and shrinks some things, and swells and vacuolates others. But notwithstanding this it is frequently very convenient on account of its *compatibility* with the most *various stains*. It has a high degree of *penetration*, and is a valuable ingredient in many *mixtures*.

It is a powerful reducing agent, and therefore *incompatible* with such reagents as chromic acid or osmic acid and the like, which it very rapidly decomposes.

For *fixing* I find that a strength of about 4 per cent. (1 vol. formol to 9 of water, or to 8 of water if the formol has been long kept) is generally about right; and this is the strength used by most writers. MAYER takes 1 of formol to 9 of sea-water, for marine animals. Few workers use much stronger solutions. Only one (HOYER, *Anat. Anz.*, ix, 1894, p. 236, Erganzungsheft) seems to have used concentrated solutions. I think this exaggerated, for I have found enormous over-fixation with solutions of 1 to 2 vols. of water. Wash out with alcohol (of 50 per cent. or more), not water.

For *hardening*, the same strengths may be taken. Hardening is more rapid than with alcohol. For prolonged hardening, considerable volumes of liquid should be taken, and the liquid should be renewed from time to time; for the formaldehyde fixes itself on the tissues with which it comes in contact, deserting the solution, which thus becomes progressively weaker. The specimens should be suspended in the liquid or otherwise isolated from contact with the containing vessel. The hardening obtained is gentle and tough, giving an elastic and not a brittle consistency. It varies greatly with different tissues. Mucin is not precipitated and remains transparent. Fat is not dissolved. Micro-organisms retain their specific staining reactions. Formaldehyde is said to harden celloidin as well as gelatin, and to be useful for celloidin-imbedding (Blum, *Anat. Anz.*, xi, 1896, p. 724).

Several of the following mixtures are irrational, becoming reduced more or less quickly, but may give good results all the same.

109. **Alcoholic Formol** (Lavdowsky, *Anat. Hefte*, iv, 1894, p. 361.)—Water 40 parts, 95 per cent. alcohol 20, formol 6, acetic acid 1; or water 30, alcohol 15, formol 5, acetic acid 1.

Gulland (*Zeit. wiss. Mikr.*, xvii, 1900, p. 222) takes (for blood) 1 part formol and 9 parts of alcohol.

Bles (*Trans. Roy. Soc. Edinburgh*, xli, 1905, p. 792) takes 1 of formol, 90 of alcohol of 70 per cent., and 3 of acetic acid.

Tellyesniczky (*Encycl. mikr. Techn.*, i, p. 472) takes 5 of formol, 100 of alcohol of 70 per cent., and 5 of acetic acid.

110. **Picro-Formol.**—P. Bouin (*Phénomènes cytologiques anormaux dans L'Histogenèse*, etc., Nancy, 1897, p. 19) recommends—

Picric acid, saturated aqueous sol.	75 parts.
Formol	25 „
Acetic acid	5 „

Wash out with alcohol, first of 50 per cent., then 70 per cent. till the picric acid is mostly removed. I consider this to be for most purposes the most valuable fixative yet made known. I have satisfied myself that the proportions are exactly what they should be and cannot be changed without hurt. It is rather a strong fixative, and should not be allowed to act for more than 18 hours. If a weaker mixture be desired, dilute the whole with water. The penetration is great, the fixation equable, delicate detail well preserved, staining qualities admirable, especially with iron-hematoxylin and Saürefuchsin. See also Garnier, *Bibl. Anat.*, v, 1898, p. 279.

The formulæ of Graf (*State Hosp. Bull. New York*, 1897; *Journ. Roy.*

Mic. Soc., 1898, p. 492) are in my view too weak, and suffer by the omission of the acetic acid.

Moreaux (*Bibl. Anat.*, 1910, p. 265) takes 15 parts formol, 85 of trichlor-acetic acid of 3 per cent., and picric acid to saturation.

111. Picro-platinic Formol (M. and P. Bouin, *Bibl. Anat.*, 1898, f. 2, p. 2).—

Platinum chloride, 1 per cent. sol.	20 parts.
Picric acid, saturated sol.	20 „
Formol	10 „
Formic or acetic acid	5 „

I find this excellent, but the mixture does not keep more than a day or two.

Bouin also (*Arch. Biol.*, xvii, 1900, p. 211) simply substitutes formol for the osmic acid in Hermann's mixture, § 45.

112. Sublimate Formol (M. and P. Bouin, *loc. cit.*).—A similar mixture, in which sublimate of 1 per cent. is substituted for the platinum chloride.

Another formula of the same authors (*Arch. Biol.*, xvii, 1900, p. 211) is 1 part of formol to 3 of saturated aqueous sublimate. Rinse with water and bring into alcohol of 70 per cent.

Spuler (*Encycl. mik. Technik.*, 1st ed., p. 1280) adds to sublimate of 3 per cent. or more 1 per cent. of glacial acetic acid and 10 per cent. of formol.

Mann (*Verh. Anat. Ges.*, 1898, p. 39) takes for nerve-cells 2½ g. sublimate, 1 g. picric acid, 5 c.c. formol, and 100 c.c. water, or (*Methods*, etc., p. 97) for all tissues 2½ g. sublimate, 20 c.c. formol, and 80 c.c. water.

Branca (*Journ. Anat. et Phys.*, xxxv, 1899, p. 767) adds 10 parts of formol and 1 of acetic acid to 60 parts of saturated solution of picric acid in saturated aqueous sublimate.

Nowak (*Anat. Anz.*, xx, 1901, p. 244) takes 30 parts of saturated sublimate, 30 of 1 per cent. chromic acid, 27 of water, 3 of acetic acid, and 10 of formalin.

113. Formol-Müller.—This is the name given by Orth (*Berl. klin. Wochenschr.*, 1896, No. 13) to a mixture of 1 part of formol with 10 of liquid of Müller (§ 53). It should be freshly made up. Fix for three hours in the stove, or twelve at normal temperature, wash out with running water. Much used, especially for nervous tissues.

Moeller (*Zeit. wiss. Zool.*, lxvi, 1899, p. 85) takes 1 vol. of formol and 4 of 3 per cent. bichromate (for the intestine of mammals).

Held (*Abh. Sächs. Ges. Wiss.*, xxxi, 1909, p. 196) takes 3 per cent. sol. of bichromate with 4 per cent. of formol and 5 per cent. of acetic acid (for inner ear). See also Morel and Bassal, *Journ. Anat. Phys.*, xlv, 1909, p. 632.

114. Chromic Acid Formol.—Lo Bianco fixes marine animals for half to one hour in 10 parts of 1 per cent. chromic acid with 1 of formol and 9 of sea-water, and passes into graded alcohols.

Marchoux (from Pérez, *Arch. Zool. Expér.*, v, 1910, p. 11) takes 11 parts 1 per cent. chromic acid, 1 of acetic acid, 4 of water, and 16 of formol (added just before using).

115. Copper Formol.—Nelis (*Bull. Acad. Sc. Belg.*, 1899 [1900], p. 726) fixes spinal ganglia for twenty-four hours in 1 litre of 7 per cent. formol with 5 c.c. of acetic acid, 20 g. of cupric sulphate, and sublimate to saturation.

Stappers (*La Cellule*, xxv, 1909, p. 356) used (for Sympoda) a mixture of Gilson's: 100 parts of formol of 5 per cent. with 2 of nitrate of copper.

Strong (*Journ. Comp. Neur.*, xiii, 1903, p. 296) fixes the head of *Acanthias* by injecting a mixture of equal parts of formol and 5 per cent. solution of bichromate of copper.

116. Nitric Acid Formol.—Wilhelmi (*Fauna u. Flora Golf. Neapel*, xxxii, 1909, p. 15) fixes Triclads in Apáthy's mixture of equal parts of 6 per cent. nitric acid and 6 per cent. formol, and brings them direct into strong alcohol.

117. Acetone Formol.—Bing and Ellermann (*Arch. Anat. Phys. Phys. Abth.*, 1901, p. 260) fix medullated nerves in 9 parts of acetone with 1 of formol.

CHAPTER VI.

DE-ALCOHOLISATION AND CLEARING AGENTS.

118. Introduction.—*De-alcoholisation agents* are liquids employed for the purpose of getting rid of the alcohol which has been employed for dehydrating tissues (§ 3), and facilitating the penetration of the paraffin used for imbedding, or the balsam or other resinous medium in which preparations are, in most cases, finally mounted. Hence all of them must be capable of expelling alcohol from tissues, and must be at the same time solvents of Canada balsam and the other resinous mounting media. The majority of them are essential oils.

Clearing agents are liquids whose function it is to make microscopic preparations transparent by penetrating amongst the highly refracting elements of which the tissues are composed, the clearing liquids themselves having an index of refraction superior, or equal, or, at all events, not greatly inferior to that of the tissues to be cleared. Hence all clearing agents are liquids of high index of refraction.

The majority of de-alcoholisation agents being also liquids of high refraction, it follows that they serve at the same time for de-alcoholisation and for clearing; and in consequence it has come about that de-alcoholisation agents are generally spoken of as clearing agents. But that practice is not strictly correct, for not all clearing agents are solvents of the resins, and not all de-alcoholising agents can serve as clearers. I shall, however, still in many cases continue to use the term "clearing" to signify "de-alcoholising," for the sake of brevity.

Neelsen and Schiefferdecker (*Arch. Anat. Phys.*, 1882, p. 206) examined a large series of ethereal oils (prepared by Schimmel and Co., Leipzig), with the object of finding a not too expensive substance that should combine the properties of clearing quickly alcohol preparations, *not* dissolving out anilin colours, clearing celloidin without dissolving it, and not evaporating too quickly.

Of these, the following three fulfil the conditions:—*Cedar-wood, Origanum, Sandal-wood.*

To these should be added the others recommended in the following paragraphs.

See also the paper of Jordan (*Zeit. wiss. Mik.*, xv, 1898, p. 50), as to the behaviour of some essential oils towards celloidin.

119. The Practice of De-alcoholisation or Clearing.—The old plan was to take the object out of the alcohol and float it on the surface of the de-alcoholising or clearing medium in a watch-glass. This plan was faulty, because the alcohol escapes from the surface of the object into the air quicker (in most instances) than the de-alcoholising or clearing agent can get into it; hence the object must shrink. To avoid this cause of shrinkage, the operation is now generally done by the method suggested by Mayer and Giesbrecht, which consists in putting the clearing medium *under* the alcohol containing the object, as described § 5. The objects should not be considered to be perfectly penetrated by the clearing medium until the wavy refraction-lines caused by the mixture of the two liquids at their surface have ceased to form, and they should not be mounted or imbedded until they have first been soaked for some time in a fresh quantity of clearing medium, to remove any alcohol that has got into the first bath.

The penetration of all clearing media may be hastened by using them *warm.*

It frequently happens that the essential oil with which objects are being treated in a watch-glass or on a slide becomes cloudy after a short time, and fails to clear the tissues. This is owing to a combination between the essential oil and moisture, derived, I think, rather from the air than from the objects themselves. The cloudiness can usually be removed by warming (as pointed out by Hatchett Jackson, *Zool. Anzeig.*, 1889, p. 630), but in certain moist states of the atmosphere it may persist, notwithstanding continued warming. It is for this reason that I advise that clearing be done, whenever possible, in shallow *corked tubes*, under which conditions the phenomenon rarely occurs. In any case, be careful not to breathe on the liquid.

120. Choice of a De-alcoholisation or Clearing Agent.—I advise the beginner to keep on his table the following:—Oil of cedar, for general use and for preparing objects for imbedding in paraffin; clove oil for making minute dissections in (§ 122), and for much work with safranin, etc.; oil of bergamot, which will clear from 90 per cent. alcohol, and which does not extract coal-tar colours; carbolic acid, for rapidly clearing very imperfectly dehydrated objects.

For special clearers for *celloidin sections* see Chapter IX.

121. Cedar Oil (Neelsen and Schiefferdecker, *loc. cit.*, § 118).—Clears readily tissues in 95 per cent. alcohol *without shrinkage*; does not extract anilin colours. Celloidin sections are cleared in five to six hours.

The observer should be careful as to the quality of the cedar oil he obtains. I have examined the clearing properties of a sample, obtained from a celebrated firm, which *totally* failed to clear absolute alcohol objects after many days.

Cedar oil is *very penetrating*, and for this and other reasons is, in my experience, the very best of all media for preparing objects for paraffin imbedding. I find it to be *less hurtful to cells* than any other medium known to me. Tissues may remain in it for any length of time without hurt. If it should become milky through keeping, filter.

122. Clove Oil.—Samples of clove oil of very different shades of colour are met with in commerce. It is frequently recommended that only the paler sorts should be employed in histology. Doubtless it is, in general, best to use a pale oil, provided it be pure; but it is not always easy to obtain a light-coloured oil that is pure. Clove oil passes very readily from yellow to brown with age, so that in choosing a colourless sample you run great risk of obtaining an *adulterated* sample, for clove oil is one of the most adulterated substances in commerce.

Clove oil does not easily spread itself over the surface of a slide, but has a tendency to form very *convex drops.* This property makes it a very convenient medium for making minute dissections in. It also has the property of making tissues that have lain in it for some time very *brittle.* This brittleness is also sometimes very helpful in minute dissections.

These qualities may be counteracted if desired by mixing the clove oil with bergamot oil.

This is one of the most useful of clearers. According to Behrens (*Tabellen,* 3 ed., 1898, p. 33), it will clear from alcohol of 74 per cent.

It has a high index of refraction, and clears objects *more* than balsam mounting media. It dissolves celloidin (or collodion), and therefore should not be used for clearing sections cut in that medium, without special precautions. New clove oil washes out basic tar colours more quickly than old.

123. **Cinnamon (or Cassia) Oil** greatly resembles clove oil, but is in general thinner, and is more highly refractive. An excellent medium, which I particularly recommend.

124. **Oil of Bergamot** (Schiefferdecker, *Arch. Anat. Phys.,* 1882 [Anat. Abth.], p. 206).—Clears 95 per cent. alcohol preparations and celloidin preparations quickly, and does not extract anilin colours.

Bergamot oil is, I believe, the least refractive of these essences, having a lower index than even oil of turpentine.

Suchannek (*Zeit. wiss. Mik.,* vii, 1890, p. 158) says that bleached, colourless bergamot oil will not take up much water, whereas a green oil will take up as much as 10 per cent.

Van der Stricht (*Arch. de Biol.,* xii, 1892, p. 741) says that bergamot oil will, with time, dissolve out the fatty granules of certain ova.

125. **Oil of Origanum** (Neelsen and Schiefferdecker, *Arch. Anat. Phys.,* 1882, p. 204).—Ninety-five per cent. alcohol preparations are cleared quickly, and so are celloidin sections, without solution of the celloidin. Anilin colours are somewhat extracted.

For work with celloidin sections care should be taken to obtain *Ol. Origani Cretici* ("Spanisches Hopfenöl"), not *Ol. Orig. Gallici* (v. GIESON; see *Zeit. wiss. Mik.*, iv, 1887, p. 482). Specimens of origanum oil vary greatly in their action on celloidin sections.

SQUIRE, in his *Methods and Formulæ*, etc., p. 81, says that origanum oil (meaning the commercial product) is nothing but oil of white thyme more or less adulterated (see next §), and that the product sold as Ol. Origani Cretici is probably oil of marjoram.

126. Oil of Thyme.—FISH (*Proc. Amer. Mic. Soc.*, 1893; *Zeit. wiss. Mik.*, xi, p. 503), following BUMPUS, says that for most of the purposes for which origanum oil has been recommended, oil of thyme will do just as well if not better. The *red* oil is just as efficient as the *white* for clearing.

Schimmel and Co., in their Report of October, 1895, p. 69, state that in France white oil of thyme is adulterated with oil of turpentine to the extent of as much as 50 per cent.

127. Oil of Gaultheria.—Used by UNNA (*Monatschr. prakt. Derm.*, *Ergänzungsh*, 1885, p. 53) for thinning balsam. The artificial oil, **methyl salicylate**, is recommended by GUÉGUEN (*Comp. Rend. Soc. Biol.*, v, 1898, p. 285) both as a de-alcoholisation and clearing agent and as a solvent of paraffin. The refractive index is 1·53. It is, unfortunately, very sensitive to water.

128. Sandal-wood Oil (NEELSEN and SCHIEFFERDECKER, *loc. cit.*).—Very useful, but its high price is prohibitive.

129. Oil of Cajeput.—Now much used. I have used it myself and found it to clear well, but to be rather thin. CARNOY and LEBRUN (*La Cellule*, xiii, 1897, p. 71) have found it useful for clearing celloidin sections. It dissolves celloidin very slowly and clears without shrinkage.

130. Oil of Turpentine.—Generally used for dissolving out the paraffin from sections; but many other reagents, such as xylol, benzol, are preferable for this purpose. If used for alcohol objects, it causes considerable shrinkage, and *alters the structure of cells* more than any other clearing agent known to me. Turpentine has, I believe, the lowest index of refraction of all the usual clearing agents except bergamot oil; it clears objects *less* than balsam.

131. Terpinol (liquid, from Schimmel and Co.) is recommended by Mayer, *Zeit. wiss. Mikr.*, xxvi, 1910, p. 523. Clears from alcohol of 90 per cent., or even 80 per cent.

132. **Carbolic Acid.**—Best used in concentrated solution in alcohol. Clears instantaneously, even very watery preparations. This is a very good medium, but it is better avoided for preparations of soft parts which it is intended to mount in balsam, as they generally shrink by exosmosis when placed in the latter medium. It is, however, a good medium for celloidin sections.

Gage's **Mixture** (*Proc. Amer. Soc. Micr.*, 1890, p. 120).—Carbolic acid crystals melted, 40 c.c.; oil of turpentine, 60 c.c.

133. **Creosote.**—Much the same properties as carbolic acid. *Beech-wood* creosote is the sort that should be preferred for many purposes,—amongst others, for clearing celloidin sections, for which it is a very good medium.

134. **Anilin Oil.**—Common anilin oil will *readily clear sections from* 70 *per cent. alcohol,* and with certain precautions (for which see the paper of Suchannek quoted below) objects may be cleared from watery media without the intervention of alcohol at all. This renders it valuable in certain cases as a medium for preparing for paraffin imbedding. For ordinary work the usual commercial anilin will suffice; and it is immaterial whether it be colourless or have become brown through oxidation. For difficult work it is well to use a perfectly anhydrous oil. For directions for preparing this see Suchannek, *Zeit. wiss. Mik.*, vii, 1890, p. 156, or the third edition of this work.

Anilin is chiefly used for clearing celloidin sections. It ought however to be soaked out before mounting by something else (chloroform or xylol for instance for some hours), as if not removed it will brown both the tissues and the mounting medium.

135. **Xylol, Benzol, Toluol, Chloroform.**—Too volatile to be recommendable as clearing agents in which it is desired to *examine* specimens, but very useful for preparing paraffin sections for balsam. Of the three first-mentioned liquids, benzol is the most volatile, then toluol, and xylol is the least volatile, in the proportion of 4 : 5 : 9 (Squire, *Methods and*

Formulæ, p. 20). Chloroform is injurious to some delicate stains, but is in other respects an excellent de-alcoholisation agent, as it will take up a good deal of water, if any be left in the preparations. I consider it too volatile to be safe to use before balsam. Xylol is the best of these in that respect.

Both xylol and toluol are liable to become acid if kept in only partially filled vessels.

CHAPTER VII.

IMBEDDING METHODS—INTRODUCTION.

136. Imbedding Methods.—The processes known as Imbedding Methods are employed for a twofold end. Firstly, they enable us to surround an object, too small or too delicate to be firmly held by the fingers or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the composite body thus formed, the included object may be cut into sufficiently thin slices without distortion. Secondly, they enable us to fill out with the imbedding mass the natural cavities of the object, so that their lining membranes or other structures contained in them may be duly cut *in situ*; and, further, they enable us not only to surround with the supporting mass each individual organ or part of any organ that may be present in the interior of the object, but also to fill with it each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that in the thin slices cut from the mass all the minutest details of structure will precisely retain their natural relations of position.

These ends are usually attained in one of two ways. Either the object to be imbedded is saturated by soaking with some material that is liquid while warm and solid when cold, which is the principle of the processes here called *Fusion Imbedding Methods*; or the object is saturated with some substance which whilst in solution is sufficiently fluid to penetrate the object to be imbedded, whilst, after the evaporation or removal by other means of its solvent, it acquires and imparts to the imbedded object sufficient firmness for the purpose of cutting. The methods founded on this principle are here called *Evaporation Imbedding Methods*.

In any of these processes the material used for imbedding is technically termed an "imbedding mass."

There are two chief methods of imbedding—the paraffin method and the celloidin or collodion method.

The paraffin method is the one in most use; for it is the *more rapid,* requiring only hours where the celloidin process requires days or weeks; and it is the one which the most readily affords very thin sections. But this only applies to fairly small objects: with objects of much over half an inch in diameter you cannot easily get with paraffin much thinner sections than you can with celloidin; and if you try to cut in paraffin objects of still greater size, say an inch and upwards, it will frequently happen that you will not get perfect sections at all, blocks of paraffin of this size having a tendency to *split* under the impact of the knife. This defect is, however, much reduced by the employment of a softer paraffin than is usual. In this way STRASSER (*Zeit. wiss. Mik.,* ix, 1892, p. 7) has obtained series of frontal sections 30 μ thick through the entire human brain, in paraffin blocks measuring 10 × 15 cm. And MAYER, with the Tetrander microtome, has obtained series of only 7·5 μ with a surface of 4½ × 3 cm.

For very large objects celloidin is safer, because it does not split, and presents advantages for the manipulation of the sections obtained. For all classes of objects it has the advantages of affording a *transparent* mass (which facilitates orientation of the object), and of producing *less shrinkage* than paraffin (paraffin unavoidably shrinks on cooling to at least 12 per cent.). It is for these two reasons that celloidin is so frequently preferred by embryologists—even for small objects.

Aqueous masses, such as gum or gelatin, may render great service in cases in which it is desired to *avoid dehydrating* tissues, and to apply *chemical tests* to them.

137. Imbedding Manipulations.—Imbedding in a melted mass, such as paraffin, is performed in one of the following ways. A little tray or box or thimble is made out of paper, some melted mass is poured into it, and the object placed in the midst of it. Or, the paper tray being placed on cork, the object may be fixed in position in it whilst empty by means of pins and the tray filled with melted mass at one pour. The pins are removed when the mass is cold.

In either case, when the mass is cold the paper is removed from it before cutting.

To make **paper trays** proceed as follows. Take a piece of stout paper or thin cardboard, of the shape of the annexed figure (Fig. 1); thin (foreign) post-cards do very well indeed. Fold it along the lines *a a′* and *b b′*, then along *c c′* and *d d′*, taking care to fold always the same way.

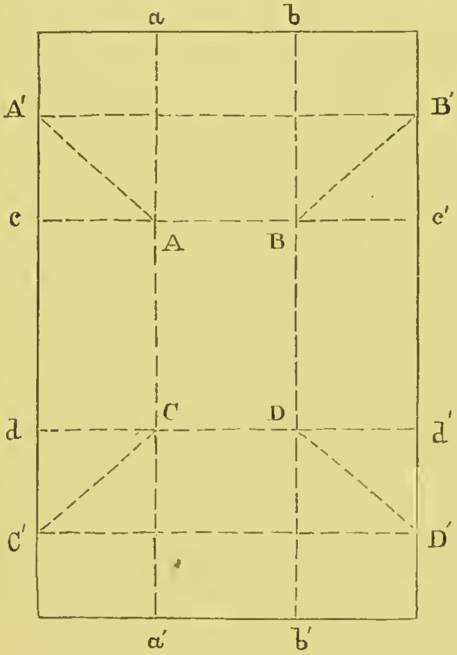

Fig. 1.

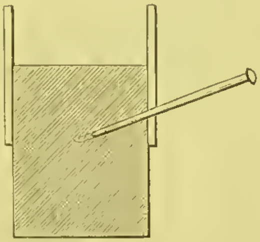

Fig. 2.

Then make the folds *A A′*, *B B′*, *C C′*, *D D′*, still folding the same way. To do this you apply *A c* against *A a*, and pinch out the line *A A′*, and so on for the remaining angles. This done, you have an imperfect tray with dogs' ears at the angles. To finish it, turn the dogs' ears round against the ends of the box, turn down outside the projecting flaps that remain, and pinch them down. A well-made post-card tray will last through several imbeddings, and will generally work better after having been used than when new.

Another method of folding the paper (Mayer) is described in the *Grundzüge*, Lee and Mayer, 4th ed., p. 77.

GIESBRECHT now makes trays of photographic films, which being transparent facilitate orientation under the dissecting microscope.

To make **paper thimbles**, take a good cork, twist a strip of paper several times round it so as to make a projecting collar, and stick a pin through the bottom of the paper into the cork. For work with fluid masses, such as celloidin, the cork may be loaded at the bottom by means of a nail or piece of lead, to prevent it from floating when the whole is thrown into spirit or other liquor for hardening (Fig. 2).

LEUCKHART's **Imbedding Boxes** are made of two pieces of type-metal (Fig. 3). Each of these pieces has the form of a carpenter's "square" with the end of the shorter arm triangularly enlarged outwards. The box is constructed by placing the two pieces together on a plate of glass which has been wetted with glycerine and gently warmed. The area of the box will vary according to the position given to the pieces, but the height can be varied only by using different sets of pieces. Two sets will be sufficient for most work; one set of one centimetre in height, and one of two centimetres, each being eight centimetres in length, and three in breadth. To make the box paraffin-tight, so that it will hold the melted paraffin long enough in the liquid state to permit of the objects being carefully orientated in it, MAYER (*Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429) first smears the glass plate with glycerin, then arranges the metal "squares," and then fills the box with collodion, which is poured out again immediately. As the ether evaporates, a thin layer of collodion remains behind, which suffices to keep the paraffin from running out. Even without the collodion, the mere cooling of the paraffin by the metal will generally suffice to keep it in long enough for orientation, if it is not in a superheated state when it is poured in.

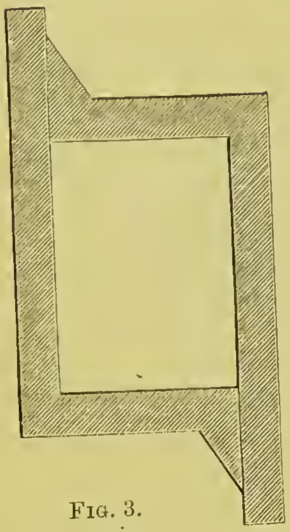

FIG. 3.

In such a collodionised box the paraffin may be kept in a liquid state by warming now and then over a spirit lamp, and small objects be placed in any desired position under the microscope (*Journ. Roy. Mic. Soc.* [N.S.], ii, p. 880).

A lighter form of "squares," made of brass, and devised by ANDRES, GIESBRECHT, and MAYER, is described *loc. cit.* (see *Journ. Roy. Mic. Soc.*, 1883, p. 913). A more complicated sort is described by WILSON in *Zeit. wiss. Mik.*, xxvii, 1910, p. 228, for use with imbedded threads to serve as orientation guides (see "Orientation").

FRANKL (*Zeit. wiss. Mik.*, xiii, 1897, p. 438) builds up boxes with rectangular blocks of glass, which may be found convenient, but are more expensive than the metal squares.

SELENKA has described and figured another sort of apparatus having the same object. It consists of a glass tube, through which a stream of warm water may be passed and changed for cold as desired, the object being placed in a depression in the middle of the tube (see *Zool. Anz.*, 1885, p. 419). A simple modification of this apparatus, which anyone may make for himself, is described by ANDREWS in *Amer. Natural.*, 1887, p. 101; and a more complicated imbedding and orienting box, seldom necessary, is described by JORDAN, in *Zeit. wiss. Mik.*, xvi, 1899, p. 32.

To imbed in a **watch-glass**, the object, previously saturated with paraffin, is put into a (preferably very concave) watch-glass containing molten paraffin. After this has been solidified by cooling (see next chapter), a block containing the object is cut out of it, and mounted on the object-holder of the microtome (this is, of course, *applicable to other masses*, such as celloidin).

For imbedding *very small objects* in this way certain precautions may be necessary in order not to lose them. SAMTER (*Zeit. wiss. Mik.*, xi, 1894, p. 469) saturates small unstained objects with paraffin that has previously been strongly coloured with alkanna extract, and then imbeds them in pure paraffin. RHUMBLER (*ibid.*, xii, 1895, p. 312, and xiii, 1896, p. 303) stains previously the objects themselves with eosin dissolved in strong alcohol, and removes the stain from the sections with weak alcohol. See also *ibid.*, xiii, p. 200, a paper by SCHYDLOWSKI; and in *Zeit. wiss. Zool.*, lviii, 1897, p. 144, a process of BORGERT.

BORGERT (*Zeit. wiss. Zool.*, lviii, 1897, p. 144) allows paraffin to solidify in a watch-glass, bores a hole in it, and places the objects in the hole with a little benzol, and puts the whole for a short time into a stove.

A watch-glass provided at the bottom with a groove or trough, in which small objects may be made to collect, is described by LEFÈVRE, *Journ. App. Mic.*, v, 1902, p. 280 (see *Journ. Roy. Mic. Soc.*, 1903, p. 233).

LAUTERBORN (*Zeit. wiss. Zool.*, lix, 1895, p. 170) brings the objects through chloroform into paraffin in a small glass tube, and after cooling breaks the tube and so obtains a cylinder of paraffin with the objects ready for cutting.

HOYER (*Arch. mik. Anat.*, liv, 1899, p. 98) performs all the operations in a glass cylinder (5 cm. long and 7 mm. wide), open at both ends, but having a piece of moist parchment paper tied over one of the openings. It is then not necessary to break the cylinder; by removing the parchment paper the paraffin can be pushed out of it in the shape of a cylinder containing the objects imbedded at one end of it.

MAYER (*Zeit. wiss. Mikr.*, xxiv, 1907, p. 130) takes the gelatin capsules used by chemists; after cooling in water the gelatin swells and is easily removed.

MEVES (*Arch. mikr. Anat.*, lxxx, Abth. ii, 1912, p. 85) employs wedge-shaped capsules made by G. Pohl, Schönbaum, Bez, Dantzig.

CHAPTER VIII.

IMBEDDING METHODS—PARAFFIN AND OTHER FUSION MASSES.

138. Saturation with a Solvent.—The first stage of the paraffin method consists in the saturation of the object with some substance which is a solvent of paraffin. The process is sometimes called "clearing," since many of the substances used for infiltration are also "clearing" agents.

The process of saturation should be carefully performed with well-dehydrated objects in the manner described in § 119.

Saturation liquids being liquids that are, on the one hand, miscible with alcohol, and on the other hand good solvents of paraffin, are not quite as numerous as could be wished.

According to GRAEFE (*Chem. Centralb.*, 1906, p. 874), at 20° C. petroleum ether (1 c.c.) dissolves 200 mg. of paraffin; chloroform 246; benzol 285; carbon tetrachloride 317. And according to APÁTHY, at 20° C. benzol dissolves 8 parts per cent., chloroform 10, toluol 10, xylol 12, oil of turpentine 8, cedar oil 4 to 6, bergamot oil 0·5 to 3, creosote and clove oil hardly any. Acetone, according to MAYER, dissolves hardly any.

Turpentine I do not recommend, because in my experience it is of all others the clearing agent that is the most *hurtful to delicate structures.*

Clove oil mixes very imperfectly with paraffin, and quickly *renders tissues brittle.*

Oil of bergamot mixes still more imperfectly with paraffin.

Benzol has been recommended by BRASS (*Zeit. wiss. Mik.*, ii, 1885, p. 301), and is now much used.

Toluol (or toluen) has been recommended by HOLL (*Zool. Anz.*, 1885, p. 223).

Xylol is said by M. HEIDENHAIN (*Kern und Protoplasma*, p. 114) to be a cause of shrinkage in cells. So it is, if you *use it to de-alcoholise* the specimens. But used *after oil of cedar*, or the like, it is very good, as it is one of the best of solvents of paraffin.

Chloroform is *deficient in penetrating power*, so that it requires an excessive length of time for clearing objects of any size; and it must be very thoroughly got rid of by evaporation in the paraffin bath, or by successive baths of paraffin, as if the least trace of it remains in the paraffin used for cutting it will make it soft. The process of removal requires a very long time, in some cases days. It ought therefore to be reserved for small and easily penetrable objects.

Naphtha has been recommended by WEBSTER (*Journ. Anat. and Physiol.*, xxv, 1891, p. 278).

FIELD and MARTIN (*Zeit. wiss. Mik.*, xi, 1894, p. 10) recommend a light petroleum known as "petroleum-æther." It is highly volatile, and thus a cause of shrinkage.

Sulphide of carbon has been recommended by HEIDENHAIN (*Zeit. wiss. Mik.*, xviii, 1901, p. 166) as being a very powerful solvent of paraffin. Most workers have found it to be much too disagreeable and dangerous a reagent for ordinary work, and *not necessary* even for delicate work.

Carbon tetrachloride has been recommended by PLEČNIK (*op. cit.*, xix, 1903, p. 328) and PRANTER (*ibid.*, p. 329) on the ground of not dissolving out osmium-blackened fats.

MAYER finds it no better than benzol.

As a general thesis, the best of all these are cedar oil, benzol, and chloroform.

Cedar-wood oil is, according to my continued experience, for the reasons stated by me in *Zool. Anz.*, 1885, p. 563, for general work the *very best* clearing agent for paraffin imbedding. It penetrates rapidly, preserves delicate structure better than any clearing agent known to me, does not make tissues brittle, even though they may be kept for weeks or months in it, and has the great advantage that if it be not entirely removed from the tissues in the paraffin bath it will not seriously impair the cutting consistency of the mass; indeed, I fancy it sometimes improves it by rendering it less brittle.

139. The Paraffin Bath.—The objects having been duly saturated with a solvent, the next step is to substitute melted paraffin for the saturating medium.

Some authors lay great stress on the necessity of making the passage from the saturating agent to the paraffin as gradual as possible, by means of successive baths of mixtures of solvent and paraffin kept melted at a low temperature, say 35° C. With *oil of cedar*, at all events, this is not necessary. I simply put the objects into melted paraffin kept just at its melting-point, and keep them there till they are thoroughly saturated; the paraffin being changed once or twice for fresh only if the objects are sufficiently voluminous to have brought over with them a notable quantity of clearing agent. If the objects have been for a very long time—months or years—in the cedar oil, so that this has become thick, I remove it partially or entirely by soaking in xylol (30 minutes to several hours) before putting into the paraffin. But with fresh oil of cedar I find no advantage in doing so.

Giesbrecht's method (*Zool. Anz.*, 1881, p. 484), is as follows:—Objects to be imbedded are saturated with *chloroform*, and the chloroform and objects are *gradually warmed* up to the melting-point of the paraffin employed, and during the warming small pieces of paraffin are by degrees *added to the chloroform*. So soon as it is seen that no more bubbles are given off from the objects, the addition of paraffin may cease, for that is a sign that the paraffin has entirely displaced the chloroform in the objects. This displacement having been a *gradual* one, the risk of shrinkage of the tissues is reduced to a minimum.

Mayer (*Grundzüge*, Lee and Mayer, 1910, p. 84) first saturates the objects with *benzol*, and then adds to the benzol some small pieces of paraffin, and lets them dissolve in the cold. After several hours (up to eighteen) the whole is brought in an open vessel on to the cold water-bath, the bath is then warmed gradually so as to attain a temperature of 60° C. in about two hours, and as fast as the benzol evaporates *melted paraffin is added* to it. Lastly, the paraffin is changed once before the definitive imbedding. He rarely leaves objects overnight in the water-bath.

Apáthy (*Mikrotechnik*, pp. 149, 150) first *clears with oil* *of cedar*, then brings the objects (by the process described § 119) into a *solution of paraffin in chloroform* saturated at the temperature of the laboratory. The objects remain in the chloroform-paraffin solution for from one to three hours, without warming, until all the cedar oil is soaked out of them. The whole is then warmed on the water-bath or oven to a few degrees above the melting-point of the paraffin intended to be used for imbedding, and the object is brought into a mixture of equal parts of paraffin and chloroform, being suspended therein *near the top* on a bridge made of hardened filter paper (or in a special apparatus to the same end, not yet described). It remains in this mixture, at the temperature of the oven, for one to three hours, and lastly is brought (still on the paper bridge or in the apparatus) into pure paraffin, where it remains for half an hour to two hours.

Denne (*in litt.*, 1907) points out that the objects ought *at first* to be *at the bottom* of the mixture. For this mixture is not a true solution, and the lower section of the contents of the tube is comparatively free from paraffin while the upper part is nearly pure paraffin. He moves the holder up in the tube at intervals, and the infiltration proceeds gradually with the minimum risk of shrinkage. Lastly, he removes the objects, on the holder, to the *top* of a tube of pure paraffin.

The practice of giving successive baths first of soft and then of hard paraffin, which has been frequently advised, appears to me *entirely illusory*.

It is important to keep the paraffin *dry*—that is, protected from vapour of water during the bath.

It is still more important to keep it *as nearly as possible* at *melting-point*. If it be heated for some time to a point much over its normal melting-point, *the melting-point will rise*, and you will end by having a harder paraffin than you set out with. And as regards the preservation of tissues, of course, the less they are heated the better. Overheating, as well as prolonged heating, tends, amongst other things, to *make tissues brittle*.

The *duration of the bath* must, of course, vary according to the size and nature of the object. An embryo of 2 to 3 millimetres in thickness ought to be thoroughly saturated after an hour's bath, or often less. Many workers habitually give much longer baths, I think often longer than necessary. But some objects, such as ova of Crustacea, may require three or four days. (HEIDECKE, *Jena. Zeit.*, xxxviii, 1904, p. 506; MAYER, *Grundzüge*, LEE and MAYER, 1910, p. 85; BRINKMANN, *Mitth. Zool. Stat. Neapel*, xvi, 1903, p. 367, three to five days for uterus of Selachians: MÜLLER, *Arch. mikr. Anat.*, lxix, 1906, p. 3, for lungs of mammals; POSO, *Esperienze microtechniche*, Napoli, 1910, p. 29, five to twelve days for uterus and placenta of *Homo.*) I take as a guide, generally, the length of time the object has taken to clear in the cedar oil, assuming that the *warm* melted paraffin ought to penetrate at least as quickly as the *cold* oil; and then allowing somewhat longer, say as much again, in order to be on the right side.

140. **Water-baths and Ovens.**—It is important that the paraffin should not be exposed to a moist atmosphere whilst it is in the liquid state. If a water-bath be used for keeping it at the required temperature provision should be made for protecting the paraffin from the steam of the heated water.

A very convenient apparatus for this purpose is that of Paul Mayer, or "Naples water-bath," which will be found described at p. 146 of *Journ. Roy. Mic. Soc.*, 1883, or CARPENTER'S *The Microscope*, p. 452. An extremely simple stove, which anyone can make for himself, is described in *Centralbl. Bakt.*, xlv, 1907, p. 191 (see *Journ. Roy. Mic. Soc.*, 1908, p. 109). For others, see the price-lists of the instrument makers, especially JUNG, and GRÜBLER and HOLLBORN; and the descriptions in the technical journals.

141. **Imbedding IN VACUO.**—There are objects which, on account of their consistency or their size, cannot be penetrated by paraffin in the ordinary way, even after hours or days in the bath. For such objects the method of imbedding *under a vacuum* (strictly, under diminished atmospheric pressure) renders the greatest service. It not only ensures complete penetration in a very short time—a few minutes—but it has the further advantage of *preventing any falling in of the tissues*, such as may easily happen with objects possessing internal cavities if it be attempted to imbed them in the ordinary way. It is realised by means of any arrangement that will allow of keeping paraffin melted under a vacuum.

That of HOFFMANN is described and figured at p. 230 of *Zool. Anz.*, 1884. In this arrangement the vacuum is produced by means of a pneumatic water aspiration pump, the vessel containing the paraffin being placed in a desiccator heated by a water bath and furnished with a tube that brings it into communication with the suction apparatus.

Francotte (*Bull. Soc. Belg. Mic.*, 1884, p. 45) produces the requisite vacuum by the condensation of steam.

Fol (*Lehrb.*, p. 121) employs the vacuum apparatus of Hoffmann, but simplifies the arrangement for containing the paraffin. The paraffin is contained in a stout test-tube furnished with a rubber stopper traversed by a tube that puts it into communication with the pump. The lower end of the test-tube dips into a water bath. You pump out the air once or twice, wait a few minutes, then turn out the object with the paraffin (which by this time will have become abnormally hard), and re-imbed in fresh paraffin.

See also Pringle, in *Journ. Path. and Bacteriol.*, 1892, p. 117; or *Journ. Roy. Mic. Soc.*, 1892, p. 893; Kolster, in *Zeit. wiss. Mik.*, xviii, 1901, p. 170; Berg, *Zeit. wiss. Mik.*, xxvi, 1909, p. 209; Fuhrmann, *ibid*, xxi, 1904, p. 462; Kolmer and Wolff, *ibid.*, xix, 1902, p. 148; Gemmill, *Journ. Roy. Mic. Soc.*, 1911, p. 26.

142. Imbedding and Orientation.—*As soon as* the objects are thoroughly saturated with paraffin they should be *imbedded* by one of the methods given above (§ 137), and the paraffin cooled as described next §.

But it may be desirable to have the object fixed in the cooled paraffin in a precisely arranged position, and, above all, in a precisely *marked* position. Very small objects may be oriented as follows:—The object is removed from the melted paraffin, and placed on a cylinder of solid paraffin. A needle or piece of stout iron wire is now heated in the flame of a lamp, and with it a hole is melted in the end of the cylinder; the specimen is pushed into the melted paraffin, and placed in any desired position. The advantages of the method lie in the quickness and certainty with which it can be performed. In using the needle it is important *to melt as little paraffin as possible at one time*, in order that that which is melted may cool again as rapidly as possible.

Kerr (*Quart. Journ. Micr. Sc.*, xlv, 1901, p. 4) employs an electrically heated needle.

The method of Patten (*Zeit. wiss. Mik.*, xi, 1894, p. 13) is useful when one desires to orient large numbers of small objects. You get some writing paper of the sort that is made with two sets of raised parallel lines running at right angles to each other ("linen cloth paper"). Small strips are cut from this, and at suitable intervals along them small drops of a mixture of collodion and clove oil, of about the consistency of thick honey, are arranged close together along one of the ribs that run lengthwise. The objects to be imbedded are cleared in clove oil or oil of bergamot. They are taken one by one on the point of a knife, and after the excess of oil has been drawn off, are transferred each to a drop of the collodion mixture, in which they will stay in any required position. When half a dozen or more objects have been oriented in reference to the cross lines (which are to be parallel to the section planes) the whole thing is placed in turpentine. This washes out the clove oil and fixes the objects very firmly to the paper. The paper with the attached objects is now passed through the bath of paraffin and imbedded in the usual way. After cooling on water the block is trimmed and the paper peeled off, leaving the objects in the paraffin close to the under-surface of the block. This surface is now seen to be marked by the orienting lines of the ribbed paper, and also by any record numbers which may before imbedding have been written with a soft pencil on the paper.

KNOWEN (*Journ. Morph.*, xvi, 1900, p. 507) takes *smooth* paper and engraves parallel lines on it with a needle, and takes xylol instead of turpentine.

A somewhat more complicated form of this process has been described by WOODWORTH, *Bull. Mus. Comp. Zool.*, xxxviii, vol. xxv, 1893, p. 45.

A similar process has also been described by FIELD and MARTIN in *Zeit. wiss. Mik.*, xi, 1894, p. 11, small strips of gelatin being used instead of paper.

MAYER also (*Grundzüge*, LEE and MAYER, 1910, p. 89) takes strips of photographic gelatin, and lets the collodion set in benzol.

HOFFMANN (*Zeit. wiss. Mik.*, xv, 1899, p. 312, and xvii, 1901, p. 443) takes, instead of the ribbed paper, glass slips ruled with a diamond, and completely imbeds the objects in large drops of clove oil collodion (equal parts), allowed to stand for twenty-four hours in an open vessel. The drops are caused to set in xylol. See also SAMTER, *ibid.*, xiii, 1897, p. 441; JORDAN, *ibid.*, xvi, 1899, p. 33; and PETER, *Verh. Anat. Ges.*, xiii Vers., 1899, p. 134.

ENTZ (*Arch. Protistenk.*, xv, 1909, p. 98) orients in clove oil collodion on a *cover-glass* coated with paraffin, and puts the whole into chloroform in which the mixture sets into a sheet which can be detached.

DENNE (*Journ. Appl. Mic.*, iii, 1902, p. 888) imbeds on disks of paper held at the bottom of glass tubes containing the paraffin by bent wires, by means of which a cylinder of paraffin containing the object may be lifted out as soon as cool.

WILSON (*Zeit. wiss. Mik.*, xvii, 1900, p. 169) makes orientation lines by imbedding alongside the objects strands of osmium-blackened nerve-fibres. See also a further development by Wilson, *ibid.*, xxvii, 1910, pp. 228 and 231.

143. Cooling the Mass.—Whatever method of imbedding and orientation in the molten paraffin has been employed, the important point now to be attended to is that *the paraffin be cooled rapidly.* The object of this is to prevent crystallisation of the paraffin (which may happen if it be allowed to cool slowly) and to get as homogeneous a mass as possible.

If the definitive imbedding has been done in a watch-glass, hold it on the top of cold water until all the paraffin has solidified, and then let it sink to the bottom. When thoroughly cool, cut out blocks containing the objects. If the watch-glass has been smeared with a drop of a mixture of equal parts of glycerine and water before putting the paraffin into it, the solidified paraffin will generally detach itself in a single cake and float up in a few minutes, or hours at any rate. Do *not* attempt to remove it entire by warming the bottom of the watch-glass. Similarly with the paper trays or metal imbedding boxes. Or you may put them to cool on a cold slab of metal or stone.

SELENKA cools the mass by passing a stream of cool water through the imbedding tube described above (§ 137). MAYER cools the mass in the paraffin-tight moulds (§ 137) by passing cold water through a special movable water-bath, which allows of the arrangement of the objects by transmitted light under a dissecting microscope, see *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 ; *Intern. Monatsschr. Anat. Hist.*, iv, 1887, p. 39. A complicated apparatus for the same purpose is described by MEISSNER (*Zeit. wiss. Mik.*, xviii, 1902, p. 286). Similarly, HAHN, *ibid.*, xxv, 1908, p. 184, and KAPPERS, *ibid.*, xxiv, 1907, p. 254.

The paraffin blocks with the objects are now mounted on the carrier of the microtome in position for cutting, and pared to the proper shape (next §). If any bubbles or cavities or opaque spots be present, prick with a heated needle till all is smooth and homogeneous. The same should be done if any cavities present themselves in the course of cutting. In bad cases, re-imbed.

144. Shape and Orientation of the Block of Mass to be cut.—These differ accordingly as the cutting is done with a slanting knife or a square-set knife (see next §). In the first case, the block is best trimmed to a three-sided prism, and orientated as in Fig. 4, so that the knife enters it at the angle *a* and leaves it at the angle *c*. When the section is cut it will adhere to the knife only by the angle *c*, and can thus most readily be removed by means of a brush or needle. The object itself should come to lie in the block close to the line *b c*, so that the knife at first cuts only paraffin, and that if the section begins to *roll* it may be caught and held down by a brush or section-stretcher before the object itself is reached. For the square-set knife the block is best trimmed to a four-sided prism, and orientated as in the first case, so that the knife first touches one angle, if only *isolated* sections are to be cut. But if *ribbons* (§ 148) are to be cut, the block must be orientated with one of its sides parallel to the knife-edge, and the opposite side must be strictly parallel to this one.

An object which is not approximately isodiametrical but gives a section which is wider in one direction than another should be orientated *end on*, that is, so as to present its narrowest diameter to the knife-edge: for it is in this position that it will offer the least resistance to the blade, and tend the least to make the edge bend away or dig into it. This is specially important with *longitudinal* sections of worms, *Amphioxus,* embryos of vertebrates, and the like. Most especially with a square-set knife should the narrowest diameter of the object be presented to the knife; and only when the object is particularly hard, or otherwise difficult to cut, should it be turned so as not to let the whole of that diameter be attacked at once by the knife, but only a corner of it. And as far as possible arrange that the hardest part of an object be the last to be touched by the knife.

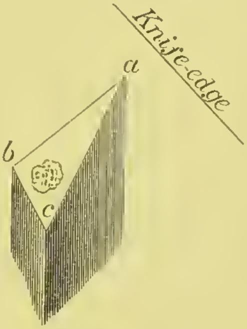

FIG. 4.

For NOACK's simple apparatus for accurately orientating small blocks, see *Zeit. wiss. Mik.*, xv, 1899, p. 438, or *Journ. Roy. Mic. Soc.*, 132, 1899, p. 550.

For ETERNOD's machine for trimming blocks to true cubes, see *Zeit. wiss. Mik.*, xv. p. 421, and for that of SCHAFFER, *ibid.*, xvi, 1900, p. 417.

145. Knife Position.—The position to be given to the knife may be considered under two heads, viz. its *slant* and its *tilt*.

By the *slant* of the knife is meant the angle that its edge makes with the line of section: that is with the line along which it is drawn through the object (or along which the object moves across it in the case of microtomes with fixed knives). The position is *transverse* when the edge makes an angle of 90° with the line of section, or the knife in that case is said to be set *square*. It is *oblique* or *slanting* when it makes a smaller angle with that line. The difference between the *effect* of the two positions is that the oblique position affords *a more acute-angled wedge* than the transverse one.

It does so for the following reasons :—Neglecting for the moment the distinction between the cutting-facets and the surfaces of the blade (which are distinct usually because they are not ground to the same angle),* it is clear that the knife itself is a wedge, the angle of which depends on the relation between the height of its base and the distance from the base to the edge. With the same base the angle becomes more acute the greater the distance from edge to base. Now by slanting the knife we can effect what is equivalent to an increase in the distance from edge to base; for we can thus increase the distance between the point of the edge which first touches the object, and the point of the back (strictly, of the back edge of the under cutting-facet) which last leaves it. When the knife is set transversely, the line along which any point of it traverses the object is the shortest possible from edge to base of the wedge, and the *effective* angle of wedge is the least acute obtainable with that knife. But if it is set as obliquely as possible, the line along which any point of it traverses the object traverses the knife from heel to toe, that is, along the greatest possible distance from edge to base, and therefore affords *practically* a much more acute-angled wedge than in the first case; and so on, of course, for intermediate positions. (See the stereometrical constructions of these relations by SCHIEFFERDECKER, *op. cit.*, p. 115; and also with more instructive figures, APÁTHY, "Ueber die Bedeutung des Messerhalters in der Mikrotomie," in *Sitzber. med.-naturw. Section d. Sieben-*

---

* The edge of a microtome knife is composed of two plane surfaces—the upper and lower cutting-facets, which meet one another at an acute angle, the cutting-edge, and posteriorly join on to the upper and lower surfaces of the blade (see some good figures of differently shaped knives in BEHRENS, KOSSEL und SCHIEFFERDECKER, *Das Mikroskop.*, p. 115, *et seq.*; and in APÁTHY'S paper quoted below). It will be seen that the two facets together form a wedge welded on to the blade by the base.

bürgischen Museumvereins, Bd. xix, Heft 7, p. 1 (Kolozsvár, 1897, A. K. Ajtai).

For *honing* knives see SSOBOLEW, *Zeit. wiss. Mik.*, xxvi, 1909, p. 65; LENDVAI, *ibid.*, p. 203; FUNCK, *ibid.*, xxvii, 1910, p. 75.

Very large objects are best cut with the slanting knife, and so are all objects of very heterogeneous consistency, such as tissues that contain much chitin or much muscular tissue; and better with a slowly working sliding microtome than with a quick-working Rocker or the like. Soft masses such as gelatin or celloidin cut wet, can *only* be cut with the slanting knife. The slanting position causes less *compression* of sections than the transverse one. It has the defect of producing rolling in paraffin sections more easily than the transverse position. The latter is the proper position for cutting *ribbons* of sections from paraffin.

By the *tilt* of the knife is meant the angle that a plane passing through its back and edge makes with the plane of section: or, practically, the greater or less degree of elevation of the back above the edge (it is not to be confounded with the inclination of the long axis of the knife to the horizon; any accidental inclination that this may have is a matter of no moment).

The question of the proper tilt to be given to the knife under different circumstances has been investigated by APÁTHY, *loc. cit. supra.* He concludes—(1) The knife should always be tilted somewhat more than enough to bring the back of the under cutting-facet clear of the object. (2) It should in general be less tilted for hard and brittle objects than for soft ones; therefore, *cæteris paribus*, less for paraffin than for celloidin. (3) The extent of useful tilt varies between 0° and 16° or occasionally 20°. (4) Excessive tilt causes rifts (longitudinal) in the paraffin, also furrows that in bad cases split up the section into narrow ribbons. It also makes sections roll. Also it may cause the knife not to bite, thus causing sections to be missed. Or it may give an undulatory surface to the sections, owing to vibrations set up in the knife, which may be heard as a deep humming tone. Further, I would add, excessive tilt may cause the knife to act as a scraper, carrying away portions of tissue bodily from their places. Excessive tilt may often be recognised by the knife giving out a short metallic sound just as it leaves the object. For knives with plane under-surfaces it is seldom advisable to give less than 10° tilt. Knives with concave under-surfaces, on the contrary, may require to be placed almost horizontal. Jung's knife-holders give mostly a tilt of about 9°, which is only enough for cutting ribbons with hard paraffin.

A knife with too little tilt will often cut a second section, or fragments of one, without the object being raised, showing that during the first cut the object was pressed down by the knife, and recovered itself afterwards. This fault is denoted by the ringing tone given out by the knife on passing *back* over the object before the latter is raised. Such a knife gives out a dull rattling sound whilst cutting. Too little tilt causes folding or puckering of sections, and does not allow of the cutting of the thinnest possible sections, as the edge does not bite enough. It is thus frequently a cause of sections being missed, or coming off thicker at one end than the other.

A slanting knife should have more tilt given to it than a square-set one.

Ribbon section-cutting (§ 148) requires a relatively hard paraffin and less tilt. With celloidin it is very important to avoid insufficient tilt, as the elastic celloidin yields before an insufficiently tilted knife and is not cut.

The tilt of the knife is given to a certain extent by the knife-holder sold with the microtome. With plane-concave knives it can be regulated to a certain extent by simply turning the blade over. It is more accurately *regulated* by means of mechanical contrivances, of which the most simple are the horseshoe-shaped wedges of Neumayer (see Jung's price-list). A pair of these, each ground to the same angle, is taken, and one of them placed (thin end towards the operator) under, and the other (thick end towards the operator) over, the clamping-arm of the knife-holder. Three pairs, having different degrees of pitch, are supplied, and are sufficient for most work. Other contrivances to the same end consist of knife-holders that permit of rotating the knife on its long axis, and, though more costly, will be found a great convenience where much section-cutting has to be done. For these see Jung's price-list, and various recent papers in *Zeit. wiss. Mik.*, also that of Apáthy, in the paper quoted above (very complicated), and especially the description of the two latest of Jung, viz. his model *l* and model *n*, by Mayer and Schoebel, in *Zeit. wiss. Mik.*, xvi, 1899, p. 29 (see figure of model *l* in *Journ. Roy. Mic. Soc.*, 132, 1899, p. 546). Also Carpenter's *The Microscope*, p. 463.

146. Cutting and Section-stretching.—Paraffin sections are cut *dry*,—that is, with a knife not moistened with alcohol or other liquid. By this means better sections are obtained, but a difficulty generally arises owing to the tendency of sections so cut to curl up on the blade of the knife. It is sometimes difficult by any means to unroll a thin section that has curled. To prevent sections from *rolling*, the following points should be attended to.

First and foremost, the paraffin *must not be too hard*, see § 151.

If, after cutting has begun, the paraffin be found to be too hard, it may be softened by placing a lamp near the imbedded object. But then, the paraffin being warmed most on the side nearest the lamp, becomes softer on that side, and the sections have a tendency to become compressed and puckered-in on that side.

If, on the contrary, the paraffin be found too soft, it may be hardened by exposing it to the cooling influence of a lump of ice.

It is often sufficient to moderate the temperature of the room by opening or closing the window, stirring the fire, setting up a screen, or the like.

For other devices for warming or cooling the paraffin see Held, *Arch. Anat. Phys., Anat. Abth.*, 1897, p. 345; van Walsem, *Zeit. wiss. Mik.*, xi, 1894, p. 218; Lendenfeld, *ibid.*, xviii, 1901, p. 18; Krause, *ibid.*, xxv, 1908, p. 299; Foot and Strobell, *Biol. Bull. Wood's Hole*, ix, 1905, p. 281.

Secondly, the knife should be set square, for the oblique position encourages rolling, and the more the knife is oblique the more do the sections roll.

Thirdly, it is better to cut ribbons than disconnected sections; ribbons of sections will often cut flat, when the same mass will only give rolled sections if cut disconnectedly.

Rolling may often be lessened or suppressed by cutting the sections thinner.

Mechanical means may be employed. The simplest of these is as follows:

During the cutting the edge of the section that begins to curl is caught and held down on the blade of the knife by means of a small camel-hair brush with a flat point, or by a small spatula made by running a piece of paper on to the back of a scalpel. Or, which is much better, the section is held down by means of an instrument called a "section-stretcher." This consists essentially of a little metallic roller suspended over the object to be cut in such a way as to rest on its free surface with a pressure that can be delicately regulated so as to be sufficient to keep the section flat without in any way hindering the knife from gliding beneath it.

See the descriptions of various forms of section-stretchers, *Zool. Anzeig.*, vol. vi, 1883, p. 100 (SCHULTZE); *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 (MAYER, ANDRES, and GIESBRECHT); *Arch. mik. Anat.*, xxiii, 1884, p. 537 (DECKER); *Bull. Soc. Belg. Mic.*, x, 1883, p. 55 (FRANCOTTE); *The Microscope*, February, 1884 (GAGE and SMITH); WHITMAN'S *Meth. in Mic. Anat.*, 1885, p. 91; *Zeit. wiss. Mik.*, iv, 1887, p. 218 (STRASSER); *ibid.*, x, 1893, p. 157 (BORN). The best are those of Mayer and Born.

I find that MAYER'S, beautifully made by JUNG, works admirably and is most valuable.

Another plan is to allow the sections to roll, but to control the rolling. To this end, the block of paraffin is pared to the shape of a wedge five or six times as long as broad, the object being contained in the broad part, and the edge turned towards the knife (see Fig. 4). The sections are allowed to roll and come off as coils, the section of the object lying in the outermost coil, which will be found to be a very open one—indeed, very nearly flat. Lay the coil on a slide with this end downwards, warm gently, and the part containing the object will unroll completely and lie quite flat.

ANILE (*Glandole duodenali*, Napoli, 1903, p. 51) and VASTARINI-CRESI (*Mon. Zool. Ital.*, 1906, p. 164) lay a strip of wet filter-paper on the block.

A defect opposite to that of the rolling of sections is the *compression* and the crumpling or puckering of sections, indicating that the paraffin has been compressed by the knife instead of being merely cut true by it. Such sections, besides showing creases or folds, have a smaller area than that of the block from which they are cut. This is a bad fault, for the compression may obliterate important cavities or efface important limits between cell-layers, etc. It may be caused by a badly cutting knife, and is very easily caused by the paraffin being too soft. To prevent it, correct the knife or cool the paraffin, or re-imbed in harder paraffin.

Very large sections tend to form folds *on the knife*, and are difficult to remove from it. MAYER (*Grundzüge*, LEE and MAYER, p. 94) gets them to wrap themselves round a glass or gelatin tube laid on the block just in front of the knife-edge and rolled forwards as it progresses. When cut, the section is rolled off on to the surface of water.

147. Cutting Brittle Objects (Collodionisation).—Some objects are by nature so brittle that they break or crumble before the knife, or furnish sections so friable that it is impossible to mount them in the ordinary way. Ova are frequently in this case. A remedy for this state of things consists in covering the exposed surface of the object just before cutting each section with a thin layer of collodion, which serves to hold together the loose parts; and will enable the operator to *cut sections considerably thinner* than can be obtained in the usual way.

The primitive form of the process was to place a drop of collodion on the free surface of each section just before cutting it. But this practice has two defects; the quantity of collodion employed sensibly softens the paraffin, and the thick layer of collodion when dry causes the sections to roll.

MARK (*Amer. Natural.*, 1885, p. 628; cf. *Journ. Roy. Mic. Soc.*, 1885, p. 738) gives the following directions:

"Have ready a little very fluid collodion in a small bottle, through the cork of which passes a small camel-hair brush, which just dips into the collodion with its tip. The collodion should be of such a consistency that when applied in a thin layer to a surface of paraffin it dries in two or three seconds without leaving a shiny surface. It must be diluted with ether as soon as it begins to show signs of doing so.

"Take the brush out of the collodion, wipe it against the neck of the bottle, so as to have it merely moist with collodion, and quickly pass it over the free surface of the preparation. Care must be taken not to let the collodion touch the vertical surfaces of the paraffin, especially not the one which is turned towards the operator, as that will probably cause the section to become stuck to the edge or under-surface of the knife. As soon as the collodion is dry, which ought to be in two or three seconds, cut the section, withdraw the knife, and pass the collodion brush over the newly exposed surface of the paraffin. Whilst this last layer of collodion is drying, take up the section from the knife and place it with the collodionised surface downwards on a slide prepared with fixative of Schaellibaum. Then cut the second section, and repeat the manipulations just described in the same order."

HENKING (*Zeit. wiss. Mik.*, iii, 1886, p. 478) takes instead of collodion a solution of *paraffin* in absolute alcohol.

For extremely brittle objects, such as ova of Phalangida, he recommends a thin (light yellow) solution of *shellac* in absolute alcohol.

HEIDER (*Embryonalentw. v. Hydrophilus*, 1889, p. 12; cf. *Zeit. wiss. Mik.*, viii, 1892, p. 509) employs a solution made by mixing a solution of *gum mastic* in ether, of a syrupy consistency, with an equal volume of collodion, and diluting the mixture with ether until quite thin and liquid.

RABL (*ibid.*, xi, 2, 1894, p. 170) employs *superheated paraffin* (of about 100° C.). This has the advantage of filling up any cavities there may be in the objects, and also of *preventing the sections from rolling*. A complicated development of this process is described by LENDENFELD in *Zeit. wiss. Mik.*, xviii, 1901, p. 18.

APÁTHY (*Mikrotechnik*, p. 183) employs a 1 per cent. solution of celloidin, allows the sections to roll, and unrolls them by the water-process (§ 149).

JORDAN (*Zeit. wiss. Mik.*) adds 5 drops of oil of cedar to 15 c.c. of the solution of celloidin, and finds that rolling is prevented.

148. Ribbon Section-cutting.—If a series of paraffin sections be cut in succession and not removed from the knife one by one as cut, but allowed to lie undisturbed on the blade, it not unfrequently happens that they adhere to one another by the edges so as to form a chain or ribbon which may be taken up and transferred to a slide without breaking up, thus greatly lightening the labour of mounting a series. For the production of a ribbon, the paraffin must be of a *melting-point* having the right relation to the temperature of the laboratory, see § 151. Secondly, the *knife should be set square*. Thirdly, the block of paraffin should be trimmed so as to present a straight edge parallel to the knife edge; and the opposite edge should also be parallel to this. It is by no means necessary to have recourse to special mechanical contrivances, as in the so-called ribbon microtomes; the Thoma microtome is sufficient. But the automatic microtomes, and amongst them the Cambridge Rocking Microtome and the Minot, are certainly most advantageous for this purpose.

If the paraffin is very hard, it is *necessary* for sections of 10 μ, and advisable for thinner ones, to *coat the block* with softer paraffin. To do this, take paraffin of about 40° C. melting-point, melt it, heat it to about 80° on the water-bath, dip the block into it for an instant, and rapidly turn it over so that the fluid paraffin may run down away from the top part as much as possible. Allow it to cool, and pare away again the soft paraffin from the two sides that are not to be arranged parallel to the knife. Or, as I frequently prefer, simply plaster a wall of soft paraffin (superheated) on to the fore and aft faces of the block with a small spatula. Large blocks may have two coatings given them.

It sometimes happens that the ribbon becomes *electrified* during the cutting, and twists and curls about in the air in a most fantastic and undesirable manner. It may be got flat by warming slightly.

149. Section Flattening.—The sections having been obtained may be cleared and mounted at once if they are quite perfect, that is, neither rolled nor creased nor *compressed.* But, should they *in the least degree* show any of these defects, they must first be unrolled or smoothed, or *expanded* to their proper dimensions.

The most efficacious plan is combined treatment with fluid and heat. The sections are either floated on to the surface of warm water or warm alcohol contained in a suitable dish, which causes them to flatten out perfectly, and are then transferred to a slide, by floating them into position, or otherwise. Or the slide has a layer of water spread over it, the sections are laid on the water, and the slide is heated (to somewhat *below* the melting-point of the paraffin) until the sections flatten out, which happens in a few seconds.

A special water-bath for flattening sections is described by NOWAK in *Zeit. wiss. Mik.*, xii, 1896, p. 447.

150. Clearing and Mounting.—The sections having been duly smoothed by one of these processes, and duly fixed to the slide (Chapter X), unless it is desired to keep them loose, all that now remains is to get rid of the paraffin and mount or stain as the case may be. Many solvents have been recommended for this purpose:—Turpentine, warm turpentine, a mixture of four parts of essence of turpentine with one of creasote, creasote, a mixture of turpentine and oil of cloves, benzin, toluol, xylol, thin solution of Canada balsam in xylol (only applicable to very thin sections), hot absolute alcohol, naphtha, or any other paraffin oil of low boiling-point. Of these xylol and toluol are generally in most respects the best. Benzol and chloroform are too volatile for safe manipulation.

If the slide be warmed to the melting-point of the paraffin, a few seconds will suffice to remove the paraffin if the slide be plunged into a tube of xylol or toluol. For *thin* sections, 10 to 15 μ, it is *not necessary to warm* at all. The sections may be mounted direct from the xylol, or the slide may be brought into a tube of alcohol to remove the solvent for staining.

Paraffin sections *can* be stained without removal of the paraffin, so that after-treatment with alcohol can be suppressed, but this is only very exceptionally advantageous.

151. **Pure Paraffin.**—It is now almost universally admitted that pure paraffin is superior for ordinary work to any of the many mixtures with wax and the like that used to be recommended. Paraffin varies enormously in hardness according to the temperature of its surroundings. It should therefore be taken of a melting-point suitable to the temperature of the laboratory. *A paraffin melting at* 50° C. or a little harder, is that which in my experience gives the best results *so long as the temperature of the laboratory is between* 15° and 17° C. For higher temperatures a harder paraffin is required, and for lower temperatures a softer one.

Many workers of undoubted competence prefer masses somewhat harder than this; so, for instance, Heidenhain (58°), Apáthy (55°), Rabl (56°), Mayer (58° to 60° in summer; in winter about 56°, but never less than 50°). Mayer points out that at Naples the temperature during five months of the summer and autumn is over 22° C. in the laboratory, sometimes over 30°. Temperatures such as these are seldom realised in the British Isles, and, whilst I quite admit that such hard paraffin may have its *raison d'être* for Naples, I hold that for that very reason it is in general unnecessarily hard for cooler climates.

My recommendation of a relatively soft paraffin refers to work with the Thoma sliding microtome. Microtomes with *fixed* knives, such as the Cambridge, the Minot, or the Reinhold-Giltay, will give good results with much harder paraffin, and, in fact, require such.

Stout knives of hard steel will take a harder paraffin than thin ones of soft steel; but the latter may be preferable for soft masses.

For *thin sections* a harder paraffin is required than for thick ones.

Hard objects require a harder paraffin than soft ones.

Brass (*Zeit. wiss. Mik.*, ii, 1885, p. 300) recommends paraffin that has been kept for some years, as it has less tendency to crystallise than new paraffin.

Paraffin of various melting-points is easily found in commerce. Intermediate sorts may be made by mixing hard and soft paraffin. I find that two parts of paraffin melting at 50° with one of paraffin melting at 36° C. give a mass melting at 48° C., and a mixture of one part of that melting at 53° with one part of that melting at 45° gives a mass melting at 50° C.

According to E. Burchardt (*Jena Zeit. Naturw.*, xxxiv, 1900, p. 719) *mixtures* of paraffins of different melting-points give better results than an unmixed paraffin of the same melting-point as the mixture. He recommends 10 parts of 40° paraffin + 1 of 45° + 1 of 52° + 1 of 58° + 6 of 60°.

For methods for ascertaining melting-points see *Kissling, Chem. Centralb.* ii, 1901, p. 507.

152. Overheated Paraffin.—Spee (*Zeit. wiss. Mik.*, ii, 1885, p. 8) takes paraffin of about 50° C. melting-point and heats it in a porcelain capsule by means of a lamp until it has become brownish-yellow, and after cooling shows an unctuous or soapy surface on being cut. This mass may be obtained ready prepared from Grübler. The object of this preparation is to make the mass stickier, in view of cutting ribbons.

Van Walsem (*Verh. Akad. Wetensch. Amsterdam*, 1899, p. 132) still recommends the addition of 5 per cent. of yellow wax to paraffin of 52° to 57° melting-point (for large sections of central nervous system).

Johnston (*Journ. Appl. Micr.*, vi, 1903, p. 2662) adds 1 per cent. of india-rubber in very small pieces, dissolved by heating to 100° C. for twenty-four hours, or several days to 60° C. Clear with xylol. For very brittle objects.

153. Soap Masses.—These have never been much used, and are now entirely discarded. But see early editions, or PÖLZAM (*Morph. Jahrb.*, iii, 1877, p. 558); KADYI (*Zool. Anz.*, 1879, vol. ii, p. 477); DÖLLKEN (*Zeit. wiss. Mik.*, xiv, 1897, p. 32).

Gelatin Masses.

154. Gelatin Imbedding is a method that has the advantage of being applicable to tissues that have not been *in the least degree dehydrated.*

The *modus operandi* is, on the whole, the same as for other fusion masses, with the difference that the objects are prepared by saturation with *water* instead of alcohol or a clearing agent. After the cooling of the mass it may sometimes be cut at once, but it is generally necessary to harden it. This may be done by treatment for a few minutes with absolute alcohol (KAISER), or for a few days with 90 per cent. alcohol (KLEBS) or chromic acid (KLEBS) or formaldehyde (NICOLAS), or it may be frozen (SOLLAS).

The mass can be removed from the sections by means of warm water.

155. Glycerin Gelatins, KLEBS' (*Arch. mik. Anat.*, v, 1869, p. 165).—A concentrated solution of isinglass mixed with half its volume of glycerin.

KAISER'S (*Bot. Centralb.*, i, 1880, p. 25).—One part by weight of gelatin is left for about two hours in 6 parts by weight of water; 7 parts of glycerin are added, and for every 100 grm. of the mixture 1 grm. of concentrated carbolic acid. The whole is warmed for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared.

GERLACH'S (*Unters. a. d. Anat. Inst. Erlangen*, 1884; *Journ. Roy. Mic. Soc.*, 1885, p. 541).—Take gelatin, 40 grm.; saturated solution of arsenious acid, 200 c.c.; glycerin, 120 c.c. Clarify with white of egg. The objects to be prepared for imbedding by a bath of one third glycerin.

APÁTHY (*Mitth. Z. Stat. Neapel*, xii, 1897, p. 718, and *Zeit. wiss. Mikr.*, xxix, 1913, p. 472) soaks *small* objects first in glycerin and water (equal parts) and then for at least 24 hours at 40° C. in a solution of 1 part of gelatin in 3 of glycerin and 6 of water. They are then arranged in some of this in an imbedding box, and the whole is warmed (over calcium chloride) in a stove at 45° to 60° C. until the mass has evaporated down to one half, losing 5 of its 6 volumes of water (as I understand—the description is not clear). Blocks are then cut out and hardened in *absolute* alcohol (suspended therein) for several days (1 day per millimetre of thickness), cleared in terpinol (1 day per millimetre), and cut with a knife wetted with the same. Said to give sections of 3 μ, without the least shrinkage.

Brunotti's **Gold Gelatin Mass** (*Journ. de Botan.*, vi, 1892, p. 194).—Twenty grms. gelatin dissolved with heat in 200 c.c. distilled water, and 30 to 40 c.c. of glacial acetic acid with 1 grm. corrosive sublimate added after filtering. Objects are prepared by soaking in some of the mass diluted with two to three volumes of water, then imbedded in the undiluted mass. The mass is then hardened in spirit or bichromate of potash, picric acid, or the like. No heat at all is required in this process.

Nicolas's **Method** (*Bibiogr. Anat.*, Paris, 3 année, 1896, p. 274).—Preparations are first soaked for one or two days in a 3 per cent. to 4 per cent. aqueous solution of gelatin kept at 25° C., then for the same time in a 10 per cent. solution, and then for two or three days more in a 20 per cent. to 25 per cent. solution containing 8 per cent. to 10 per cent. of glycerin and kept at 35° C. They are then imbedded in some of the same mass in paper trays, and as soon as the gelatin has set are thrown into a mixture of formol 1 part, water 7. After a few days therein the gelatin has become hard and insoluble, and may be cut or preserved for months in weak formol solution, or dilute alcohol or glycerin, or even in pure water. Sections must be very gradually passed through successive alcohols for dehydration, as they curl up very easily. They, however, flatten out at once on being brought from absolute alcohol into cresylol, and may then be mounted in balsam. To mount in glycerin is of course easy.

Burzynski (*Polu. Arch. Biol. Med. Wiss.*, i, 1901, p. 39) finds that alkaline formol hardens gelatin better than acid.

Gaskell (*Journ. Path. Bact.*, July, 1912, p. 58) soaks in *pure* gelatin, melted *s.a.*, for two to five hours at 37° C., and hardens the mass in *vapour* of formol, for three or more days. To cut, he freezes. He mounts in glycerin jelly, to avoid dehydration and shrinkage.

CHAPTER IX.

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS.

156. Introduction.—Collodion (or celloidin) masses do not require the employment of heat. They do not require that the objects should be cleared before imbedding, and that is an advantage in the case of very large objects. They are more or less transparent, which facilitates orientation. And they are specially indicated for very large objects, for the soaking in collodion, being quite inoffensive to the most delicate elements, may be prolonged if necessary for weeks. Lastly, the mass being quite transparent after mounting, it is not necessary to remove it from the sections before staining and mounting them; it may remain, and fulfil the function of an admirable support to the tissues, holding in their places brittle or detached elements that without that help would fall to pieces and be lost.

There are disadvantages. One is that the process is a very long one; as usually practised, it requires some three days for the imbedding of an object that can be imbedded in paraffin in an hour. Another is that it is impossible to obtain with celloidin sections quite so thin as those furnished by paraffin.

In the *older celloidin method* the mass is cut *wet, before clearing*. I strongly recommend the more recently introduced practice of *clearing before cutting*, and *cutting dry* as described in §§ 168–170.

157. Collodion, Celloidin, and Photoxylin.—The collodion method is due to DUVAL (*Journ. de l'Anat.*, 1879, p. 185).

Celloidin, recommended later on by MERKEL and SCHIEFFERDECKER (*Arch. Anat. Phys.*, 1882, p. 200), is merely a patent collodion. It may be obtained from GRÜBLER, or the other dealers in histological reagents. It is sent out in the form of tablets. These tablets may, if desired, be dissolved at once in ether, or a mixture of ether and alcohol, to make a collodion of any desired strength. But it is better, as recommended by Apáthy, to cut them up into thin shavings, which should be allowed to dry in the air until they become yellow, transparent, and of a horny consistency, and that these be then dissolved in alcohol and ether (sulphuric, free from acid). The solutions thus prepared are *free from the excess of water* that is present in the undried celloidin, and give after hardening a mass that is *more transparent* and of a better consistency for cutting (*Zeit. wiss. Mik.*, vi, 1889, p. 164).

Imbedding masses of excellent quality can be prepared with ordinary collodion, but celloidin furnishes more readily solutions of known concentration. Otherwise there is but little to choose between the two, and therefore in this work the terms collodion and celloidin are used indifferently.

According to Unna (*Monatschr. p. Dermatol.*, xxx, 1900, pp. 422 and 476; *Zeit. wiss. Mik.*, xviii, 1901, p. 32) a more inelastic, and therefore better, mass is obtained by adding to celloidin 2 per cent. of oil of turpentine, stearate of soda, or (best of all) castor-oil. Celloidin with this addition has been put on the market under the name of "Celloidinum inelasticum," by the *Chemische Fabrik vorm. E. Schering*, in Berlin.

Photoxylin (Krysinsky, Virchow's *Archiv*, cviii, 1887, p. 217; Busse, *Zeit. wiss. Mik.*, ix, 1892, p. 47) is a dry substance, of the aspect of cotton-wool, and chemically nearly related to celloidin. It can be obtained from Grübler. It gives a clear solution in a mixture of equal parts of ether and absolute alcohol, and should be used in exactly the same way as celloidin. It has the advantage of affording a mass which after hardening in 85 per cent. alcohol remains perfectly *transparent*. Some writers say that it gives a better consistency, but others deny this (Apáthy, *e. g.*).

Tschernischeff (*Zeit. wiss. Mik.*, xvii, 1900, p. 449) recommends *Colloxylin* (10 grms. dissolved in 10 grms. of eugenol or clove oil, with the addition of 50 c.c. of ether and 1 of absolute alcohol).

The Older Celloidin Method.

158. **Preparation of Objects.**—The objects must first be *very thoroughly* dehydrated with absolute alcohol. They are then soaked till thoroughly penetrated in ether, or, which is better, in a mixture of ether and absolute alcohol. Duval (*loc. cit.*) takes for this purpose a mixture of ten parts of ether to one of alcohol; Schiefferdecker (and the majority

of workers) a mixture of equal parts of ether and alcohol; Tubby (in *Nature*, November 17th, 1892, p. 51) advises a mixture of four parts of ether and one of alcohol. Fish advises acetone, see next §. Mann (*Methods*, etc., p. 172) takes equal parts of ether and methyl alcohol. So also Pavlow, *Zeit. wiss. Mikr.*, xxi, 1904, p. 15.

This stage may be omitted if the objects are of a sufficiently permeable nature, and they may be brought direct from alcohol into the collodion bath.

159. The Collodion Bath.—The secret of success here is to infiltrate the objects first with thin solutions, then with the definitive thick one. (A thin solution may be taken to mean one containing from 4 to 6 per cent. of celloidin [dried as described in § 157]; a thick solution, one containing 10 to 12 per cent.)

If collodion be taken, the thin solutions may be made by diluting it with ether. If photoxylin or celloidin be taken, the solutions are made in a mixture of ether and absolute alcohol in equal parts.

The dried celloidin shavings dissolve very slowly in the mixture. Elschnig (*Zeit. wiss. Mikr.*, x, 1893, p. 443), has found that solution is obtained much quicker if the shavings be first allowed to swell up for twenty-four hours in the necessary quantity of absolute alcohol, and the ether be added afterwards.

Busse (*op. cit.*, ix, 1892, p. 47) gives the following proportions for the successive baths:—No. 1, 10 parts by weight of photoxylin or perfectly dried celloidin to 150 parts of the ether and alcohol mixture; No. 2, 10 parts of photoxylin or celloidin to 105 of the mixture; No. 3, 10 parts to 80 of the mixture (already-used solution may be employed for the first bath).

I generally use only two solutions: one weak one, and one strong one corresponding approximately to Busse's No. 2. His No. 3 is so thick that excessive time is required to obtain penetration by it.

Mann (*Methods*, p. 172) uses solutions of 2½ and 10 per cent.

Apáthy (Behrens, *Tabellen.*, 1898, p. 82) takes 2 per cent. and 4 per cent. for the first baths, 8 per cent. for the last.

MYERS (*Arch. Anat. Phys., Anat. Abth.*, 1902, p. 370) takes 1½ per cent., 6 per cent., and 16 per cent.

See also NEUMAYER, *Zeit. wiss. Mik.*, xxv, 1908, p. 38; DE VECCHI, *ibid.*, xxiii, 1906, p. 312; and FUHRMANN, *Zeit. wiss. Zool.*, lxxviii, 1905, p. 524.

FISH (*Journ. Appl. Microscop.*, ii, 1899, p. 323) first infiltrates with *acetone* (which he says may be used as a fixing and dehydrating agent at the same time), then with a 4 per cent. solution of pyroxylin (gun-cotton) in acetone; and, lastly, in an 8 per cent. acetone solution of the same. See for other solutions §§ 170 and 171.

The objects ought to remain in the first bath until very thoroughly penetrated;—days, even for small objects,—weeks or months for large ones (human embryos of from six to twelve weeks, for instance).

When the object is duly penetrated by the thin solution, or solutions, if more than one have been employed, it should be brought into the thickest one. This may be done (as first described in this work, 1st edit., 1885, p. 194) by allowing the thin solution to concentrate slowly (the stopper of the containing vessel being raised, for instance, by means of a piece of paper placed under it), and making up the loss from evaporation with thick solution.

APÁTHY (*Mikrotechnik*, p. 121) holds that it is preferable to transfer to fresh thick solution, as he finds that a better consistency after hardening is thus obtained.

160. Imbedding.—The objects must now, if it has not been done before, be *imbedded*—that is, arranged in position in the thick collodion in the receptacle in which they are to be hardened. For the usual manipulations see § 137. If paper thimbles be taken for imbedding, the bottoms should be made of soft wood in preference to cork, see § 165. They should be prepared for the reception of the object by pouring into them a drop of collodion, which is allowed to dry. The object of this is to prevent bubbles coming up through the wood or cork and lodging in the mass. Watch-glasses, deep porcelain water-colour moulds, and the like, also make convenient imbedding receptacles. Care should be taken to have them perfectly *dry*.

It not infrequently happens that during these manipulations bubbles make their appearance in the mass. Before proceeding with the hardening these should be got rid of by exposing the whole for an hour or two to the vapour of ether in a desiccator or other well-closed vessel. Care should be taken that the ether (which may be poured on the bottom of the vessel) does not wet the mass (BUSSE, *Zeit. wiss. Mik.*, viii, 1892, p. 467).

161. Orientation.—Celloidin being more or less transparent, it is seldom necessary to recur to special aids to orientation.

APÁTHY (*Zeit. wiss. Mik.*, v, 1888, p. 47) arranges objects on a small rectangular plate of gelatin, placed on the bottom of the imbedding-recipient. The gelatin is turned out with the mass after hardening, and cut with it. The edges of the gelatin form good orientation lines.

HALLE and BORN (*Zeit. wiss. Mik.*, xii, 1896, p. 364) use plates of hardened white ot egg, in which a shallow furrow for the reception of the objects has been cut by means of a special instrument. See also § 142.

For the complicated method of EYCLESHYMER (*Amer. Nat.*, xxvi, 1892, p. 354) see *previous editions.*

See also the article "Rekonstruction" in the *Encycl. mik. Technik.*

162. Hardening, Preliminary.—The objects being imbedded, the treatment should be as follows:—The receptacles or supports are set with the mass under a glass shade, allowing of just enough communication with the air to set up a slow evaporation. Or porcelain moulds or small dishes may be covered with a lightly fitting cover. As soon as the added thick collodion (of which only just enough to cover the object should have been taken) has so far sunk down that the object begins to lie dry, fresh thick solution is added, and the whole is left as before. (If the first layer of collodion has become too dry, it should be moistened with a drop of ether before adding the fresh collodion.) Provision should be again made for slow evaporation, either in one of the ways above indicated, or—which is perhaps better—by setting the objects under a *hermetically* fitting bell-jar, which is lifted for a few seconds only once or twice a day. I have frequently found it advantageous to set the objects under a bell-jar, together with a dish containing alcohol, so that the evaporation is gone through in an *atmosphere of alcohol.* This is especially indicated for very large objects. The whole process of adding fresh collodion and placing the objects under the required conditions of evaporation is repeated every few hours for, if need be, two or three days.

When the mass has attained a consistency such that the ball of a finger (*not* the nail) no longer leaves an impress on it, it should be scooped out of the dish or mould, or have the paper removed if it has been imbedded in paper, and be submitted to the next stage of the hardening process. (If the mass is found to be not quite hard enough to come away safely, it should be put for a day or two into weak alcohol, 30 to 70 per cent.)

163. Hardening, Definitive.—Several methods are available for the definitive hardening process. One of these is the *chloroform* method, due to VIALLANES (*Rech. sur l'Hist. et le Dév des Insectes*, 1883, p. 129).

It consists in bringing the objects into *chloroform.* In some cases a few *hours'* immersion is sufficient to give the requisite consistence. In no case have my specimens required more than three days. The collodion frequently becomes opaque on being put into the chloroform, but regains its transparency after a time.

Small objects may be hardened by chloroform *without preliminary hardening by evaporation.* All that is necessary is to expose the mass to the air for a few seconds until a membrane has formed on it, and then bring it into chloroform. If the mass is in a test-tube this may be filled up with chloroform and left for two or three days if need be. By this time the collodion mass will be considerably hardened, and also somewhat shrunk, so that it can be shaken out of the tube. It is then brought into fresh chloroform in a larger vessel, where it remains for a few more days until it is ready for cutting. But sufficient hardening is sometimes obtained in a few hours.

Good chloroform is a necessity.

The above processes are excellent, but I regard them as primitive forms of the chloroform method. I now almost always harden in *vapour of chloroform.* All that is necessary is to put the liquid mass (after having removed bubbles as directed in § 160), with its recipient into a desiccator on the bottom of which a few drops of chloroform have been poured. The action is very rapid, and the final consistency of the mass at least equal to that obtained by alcohol hardening.

The more commonly employed hardening method is the *alcohol* method. The objects are thrown into alcohol and left there until they have attained the right consistency (one day to several weeks). The bottle or other vessel containing the alcohol *ought not to be tightly closed, but should be left at least partly open.*

The strength of the alcohol is a point on which the practice of different writers differs greatly. Busse (*Zeit. f. wiss. Mikr.*, ix, 1, 1892, p. 49) has found, as I also have done, that *alcohol of about* 85 *per cent.* is the best, *both as regards the cutting consistency and the transparency* of the mass. (Care must be taken to keep masses hardened in this grade of alcohol moist while cutting, as they dry by evaporation very quickly.)

Some workers use lower grades, 70 to 80 per cent., or even lower. Apáthy (*Microtechnik*, p. 185) mentions "glycerin-alcohol," but without giving details. Blum (*Anat. Anz.*, xi, 1896, p. 724) mentions "weak spirit with formol added to it," saying that formol hardens celloidin.

Lastly, the mass may be *frozen*. After preliminary hardening by alcohol, it is soaked for a few hours in water, in order to get rid of the greater part of the alcohol (the alcohol should not be removed entirely, or the mass may freeze too hard). It is then dipped for a few moments into gum mucilage in order to make it adhere to the freezing plate, and is frozen. If the mass have frozen too hard, cut with a knife warmed with warm water.

Florman (*Zeit. wiss. Mik.*, vi, 1889, p. 184) recommends that the definitive hardening should be done without the aid of alcohol or chloroform, by simply cutting out the blocks, turning them over, and carefully continuing the evaporation process in the way described above. I described this process myself in the first edition of this work. I doubt whether it is possible in this way to carry the hardening much beyond the point attained by the chloroform or alcohol method without incurring a very undesirable degree of shrinkage.

164. Preservation.—The hardened blocks of collodion may be preserved till wanted in weak alcohol (70 per cent.), or dry, by dipping them into melted paraffin (Apáthy, *Zeit. wiss. Mikr.*, v, 1888, p. 45), or, after rinsing with water, in glycerine-jelly, which may be removed with warm water before cutting (Apáthy, *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 372).

Reference numbers may be written with a soft lead pencil on the bottom of the paper trays, or with a yellow oil pencil on the bottom of the watch-glasses in which the objects are imbedded. On removal of the paper from the collodion after hardening, the numbers will be found impressed on the collodion.

165. **Cutting.**—If the object *has not been stained* before imbedding, it may form so transparent a mass with the collodion that the arrangement of the object and sections in the right position may be rendered very difficult. It is, therefore, well to stain the collodion lightly, just enough to make its outlines visible in the sections. This may be done by adding picric acid or other suitable colouring matter dissolved in alcohol to the collodion used for imbedding, or to the oil used for clearing.

To fix a collodion block to the microtome take a piece of soft wood, or, for very small objects, pith, of a size and shape adapted to fit the holder of the microtome. Cover it with a layer of collodion, which you allow to dry. Take the block of collodion or the infiltrated and hardened but not imbedded object, and cut a slice off the bottom, so as to get a clean surface. Wet this surface first with absolute alcohol, then with ether (or allow it to dry); place one drop of *very thick* collodion on the prepared wood or pith and press down *tightly* on to it the wetted or dried surface of the block or object. Then throw the whole into weak (70 per cent.) alcohol for a few hours, or even less, or, better, into chloroform, or vapour of chloroform, for a few minutes, in order that the joint may harden.

Lindsay Johnson prefers a mixture of beeswax, 1 part; rosin, 2 parts. To use it you must get the block of celloidin perfectly dry at the bottom, then warm the object-holder slightly, if possible over a flame; drop on to it a few drops of melted cement, and press on to it the block of collodion, which will be firmly fixed as soon as the cement is cool—that is, in a few seconds.

For objects of any considerable size it is best not to use cork for mounting on the microtome, if the object-holder be a vice; for cork bends under the pressure of the holder, and the elastic collodion bends with it, deforming the object. If the object-holder be of the cylinder type, a good cork may be used; but even then, I think, wood is safer. GAGE has recommended bits of glass cylinders. JELINEK (*Zeit. wiss. Mik.*, 11, 1894, p. 237) recommends a sort of vulcanite known as "Stabilit," which is manufactured for electrical insulation purposes. It is supplied in suitable blocks by JUNG, and by GRÜBLER. Wood is liable to swell in alcohol so that it no longer fits into the object-holder. BABCOCK (*Journ. R. Micr. Soc.*, 1901, p. 339) uses a block of hard paraffin, with the surface corrugated.

Sections (from such masses as have not been cleared before cutting) are cut with a knife kept abundantly wetted with alcohol (of 50 to 85 or even 95 per cent.). APÁTHY recommends that the knife be smeared with yellow vaseline; it cuts better, is protected from the alcohol, and the mobility of the alcohol on the blade is lessened.

The knife is set in as oblique a position as possible.

Very brittle sections may be collodionised as explained § 147.

The sections are either brought into alcohol (of 50 to 85 or 95 per cent.) as fast as they are made, or if it be desired to mount them in series, they are treated according to one of the methods described below, in Chapter X.

Masses that have been cleared before cutting with cedar oil or the like may be cut *dry*, § 170.

166. Staining.—The sections may now be stained as desired, either loose, or mounted in series on slides or on paper as described in Chapter X. It is *not* in general necessary, nor indeed desirable, to remove the mass before staining, as it usually either remains colourless, or gives up the stain on treatment with alcohol. But if it be desired, the mass may be removed by treating the sections with absolute alcohol or ether.

167. Clearing and Mounting.—You may mount in glycerin without removing the mass, which remains as clear as glass in that medium.

You may mount in balsam, also, without removing the mass, which does no harm, and serves the useful purpose of holding the parts of the sections together during the manipulations. Dehydrate in alcohol of 95 or 96 per cent. (not absolute, as this attacks the collodion). NIKIFOROW (*Zeit. wiss. Mik.*, viii, 1891, p. 189) recommends a mixture of equal parts of alcohol and chloroform. Clear with a substance that does not dissolve collodion. The clearing agents most recommended are origanum oil (*Ol. Origan. Cretici*, it is said, should be taken, not *Ol. Orig. Gallici*; but see as to this reagent the remarks in § 125), bergamot oil (said to make sections shrink somewhat), oil of sandal-wood, lavender oil, oil of cedar-wood (safe and gives excellent results, but acts rather slowly), chloroform, xylol, or benzol (may make sections shrink if not well dehydrated), or Dunham's mixture of 3 or 4 parts of white oil of thyme with 1 part of oil of cloves. (As to oil of thyme, see also §§ 125, 126.)

FISH (*Proc. Amer. Mik. Soc.*, 1893) advises a mixture of one part of red oil of thyme with three parts of castor oil, the latter being added in order to counteract the volatility of the thyme oil. But later (June, 1895), writing to me, Dr. Fish says he has substituted the white oil of thyme for the red, and finds it an advantage in orientating. See also § 126.

Some specimens of clove oil dissolve collodion very slowly, and may be used, but I would not be understood to recommend it. The action of origanum oil varies much, according to the samples; some sorts do not clear the collodion, others dissolve it, others pucker it. MINOT (*Zeit. wiss. Mik.*, iii, 1886, p. 175) says that Dunham's mixture "clarifies the sections very readily, and softens the celloidin just enough to prevent the puckering which is so annoying with thyme alone."

Carbolic acid has been recommended. WEIGERT (*Zeit. wiss. Mik.*, iii, 1866, p. 480) finds that a mixture of 3 parts of xylol with 1 part of carbolic acid (anhydrous) clears well. But it must not be used with the basic anilin stains, as it discolours them. For these anilin oil may be used with xylol in the place of carbolic acid.

Anilin oil clears well (it will clear from 70 per cent. alcohol), but unless thoroughly removed the preparation becomes yellowish-brown, see § 134. See VAN GIESON, *Amer. Mon. Mic. Journ.*, 1887, p. 49, or *Journ. Roy. Mic. Soc.*, 1887, p. 519, for a review of these clearing agents.

Beech-wood creasote has been recommended (by M. Flesch).

EYCLESHYMER (*Amer. Nat.*, xxvi, 1892, p. 354) advises a mixture of equal parts of bergamot oil, cedar oil, and carbolic acid.

For oil of cajeput see § 129; and for this and other clearers see also Jordan, *Zeit. wiss. Mik.*, xv, 1898, p. 51, who recommends, amongst other things, oil of Linaloa, which remains colourless.

The Newer Celloidin Method.

168. **The New Method, by Clearing before Cutting.**—This process is due, I believe, in the first instance to E. Meyer (*Biol. Centralb.*, x, 1890, p. 508), who advised soaking blocks before cutting for twenty-four hours in glycerin. Bumpus (*Amer. Anat.*, xxvi, 1892, p. 80) advises clearing the mass, after hardening in chloroform, with white oil of thyme or other suitable clearing agent (see § 167). The knife is wetted with the clearing oil, and the same oil is employed for covering the exposed surface of the object after each cut. Similar recommendations are made by Eycleshymer (*op. cit.*, pp. 354, 563), carbolic acid, or glycerin, or the mixture given § 167, being suggested for clearing; and Gilson has for a long time past adopted the practice of clearing before cutting with cedar oil, as described in the next §.

Fish (*loc. cit.*, § 167) also advocates the practice of clearing in the mass, recommending the clearing mixture there given. Similarly Gage, *Trans. Amer. Mik. Soc.*, xvii, 1896, p. 361.

All the authors above quoted cut in the *wet* way, that is to say, with a knife wetted with the clearing liquid.

169. Gilson's **Rapid Process** (communicated, April, 1892). —The object is dehydrated, soaked in ether, and brought into a test-tube with collodion or thin celloidin solution. The tube is dipped into a bath of melted paraffin, and the collodion allowed to boil (which it does at a very low temperature) until it has become of a syrupy consistence. (It should be boiled down to about one third of its volume.) The mass is then turned out, mounted on a block of hardened celloidin, and the whole hardened in chloroform or in a mixture of chloroform and cedar oil for about an hour. It is then cleared in cedar oil (if hardened in pure chloroform: special clearing will not be necessary if it has been hardened in the mixture). It may now be fixed in the microtome and cut,

using cedar oil to wet the knife, and cover the exposed surface of the object after each cut.

This process is very much more rapid than the old process: small objects can be duly infiltrated in an hour, where days would be required by the old process. As collodion boils at a very low temperature very little heat is required, and there is no risk of the tissues suffering on that head.

170. The Dry Cutting Method.—I recommend the following as a further improvement. Infiltrate with collodion or celloidin either by Gilson's process, or by soaking in the cold in the usual way, § 159. Imbed as usual. Harden in vapour of chloroform for from one hour (generally sufficient for small objects) to overnight. This is done by putting the object (definitively imbedded in the final thick solution, but without any preliminary hardening in the air) into a Steinach's sieve-dish or into a desiccator, on the bottom of which a teaspoonful of chloroform has been poured. (The objects may remain for months in the chloroform vapour if desired.) As soon as the mass has attained sufficient superficial hardness, it is, of course, well to turn it out of its recipient, and turn it over from time to time, in order that it may be equally exposed on all sides to the action of the vapour. When fairly hard throw it into Gilson's mixture. This should be at first a mixture of one part of chloroform with one or two parts of cedar oil. From time to time more cedar oil should be added, so as to bring the mixture up gradually to nearly pure cedar oil. As soon as the object is cleared throughout, the mass may be exposed to the air, and the rest of the chloroform will evaporate gradually. The block may now either be mounted on the holder of the microtome, § 165, and cut at once, or may be preserved indefinitely without change in a stoppered bottle. *Cut dry*, the cut surface will not dry injuriously under several hours. The cutting quality of the mass is often improved by allowing it to evaporate in the air for some hours.

The hardening may be done at once in the chloroform and cedar oil mixture, instead of the chloroform vapour, but I find the latter preferable. And clearing may be done in pure cedar oil instead of the mixture, but then it will be very slow, whereas in the mixture it is extremely rapid.

Stepanow (*Zeit. wiss. Mik.*, xvii, 1900, p. 185) soaks and imbeds in a solution of celloidin in a mixture of equal parts of *ether and clove oil*, hardens in alcohol or vapour of chloroform, or in *benzol*, and cuts either wet or dry.

See also Tschernischeff, *ibid.*, p. 449.

Jordan, *ibid.*, p. 193, imbeds in a mixture of 5 parts of 8 per cent. celloidine solution with 1 of *oil of cedar*, hardens first in vapour of chloroform and then in a mixture of 5 parts of chloroform with 1 of oil of cedar, and cuts wet or dry.

171. **Double Imbedding in Collodion and Paraffin.**—This is sometimes, though rarely, employed for objects of which it is desired to have very thin sections, and which are too brittle to give good sections by the plain paraffin process.

Kultschitzky's **Method** (*Zeit. wiss. Mik.*, iv, 1887, p. 48).—After the collodion bath, the object is soaked in oil of origanum (*Oleum Origani vulg.*). It is then brought into a mixture of origanum oil and paraffin heated to not more than 40° C., and lastly into a bath of pure paraffin.

The mass may be preserved in the dry state, and may be cut dry.

Ryder (*Queen's Micr. Bull.*, 1887, p. 43; *Journ. Roy. Micr. Soc.*, 1888, p. 512) modified the process by substituting chloroform for the origanum oil.

Ide (*La Cellule*, vii, 1891, p. 347, and viii, 1, 1892, p. 114) imbeds in collodion in a tube by Gilson's process (§ 169); the collodion is boiled for forty minutes, then brought for fifteen minutes (this is for small objects) into chloroform heated to 30° C. containing one fourth part of paraffin dissolved in it, then for ten minutes into pure melted paraffin.

Field and Martin (*Bull. Soc. Zool. de France*, 1894, p. 48) make a solution of dried celloidin in a mixture of equal parts of absolute alcohol and toluene, of about the consistency of clove oil. This solution is saturated with paraffin, added in shavings at a temperature not exceeding 20° to 23° C. The tissues are prepared by soaking in some of the mixture of alcohol and toluene, and are then penetrated with the celloidin-paraffin solution. The mass is hardened in a saturated solution of paraffin in chloroform or in toluene, and is finally imbedded in pure paraffin in the usual way.

Stepanow imbeds in paraffin after clearing with benzol, last §.

Jordan, after imbedding as in last §, passes through a bath of paraffin dissolved in chloroform into pure paraffin.

Wilhelmi (*Fauna Flora Golf. Neapel*, xxxii, 1909, p. 17), following Apáthy, imbeds in celloidin, hardens in chloroform, then adds benzol to the chloroform, and passes through pure benzol (half an hour to an hour) into paraffin, and cuts dry.

Similarly, Breckner, *Zeit. wiss. Mik.*, xxv, 1908, p. 29.

Sterling (*Jena Zeit.*, 1909, p. 253) soaks for two or three days in equal parts of clove oil and collodion, puts for a couple of hours (until clear) into xylol, and imbeds in paraffin.

See also Dahlgren, *Journ. Appl. Microsc.*, 1898, p. 97; Sabussow, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 353; MEYER, *ibid.*, xiv, 1901, p. 295; MITROPHANOW, *Arch. Zool. Expér.* [3], 3, 1896, p. 617; FEDERICI, *Anat. Anz.*, xxxi, 1907, p. 602; BORDAGE, *Bull. Sci. France Belg.*, xxxix, 1905, p. 385; GANDOLFI, *Zeit. wiss. Mik.*, xxv, 1909, p. 421; MAYER, *ibid.*, xxiv, 1907, p. 132.

Other Cold Masses.

172. **Joliet's Gum and Glycerin Method** (*Arch. Zool. Expér. et Gén.*, x, 1882, p. xliii).—Pure gum arabic dissolved in water to the consistency of a thick syrup. Pour a little of the solution into a watch-glass, and add from 6 to 10 drops of pure glycerin. In the winter or in rainy weather less glycerin should be taken than in the summer or dry weather.

The object is imbedded in the mass in the watch-glass and the whole left to dry for from one to four days. When it has assumed a cartilaginous consistency, a block containing the object is cut out, turned over, and allowed to dry again until wanted for use. A stove, or the sun, may be employed for drying, but it is best to dry slowly at the normal temperature.

173. STRICKER'S **Gum Method** (*Hdb. d. Gewebcl.*, p. xxiv).—A concentrated solution of gum arabic. The object is imbedded in the gum in a paper case. The whole is thrown into alcohol, and after two or three days may be cut. The alcohol should be of about 80 per cent. (MAYER).

I have seen masses of sufficiently good consistency prepared by this simple method.

174. ROBERTSON'S **Grape-sugar Method**, see *Journ. of Anat. and Physiol.*, xxiv, 1890, p. 230.

175. HYATT'S **Shellac Method**, see *Am. M. Mic. Journ.*, i, 1880, p. 8; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 320. For sections through hard chitinous organs consisting of several pieces, such as stings and ovipositors, retaining all the parts in their natural positions.

176. BRUNOTTI'S **Cold Gelatin Mass** has been given, § 155.

*Masses for Grinding Sections.**

177. G. VON KOCH'S **Copal Method** (*Zool. Anz.*, i, 1878, p. 36).—Small pieces of the object are stained in bulk and

---

* For the manipulations of section-grinding, see CARPENTER'S *The Microscope*.

dehydrated with alcohol. A thin solution of copal in chloroform is prepared by triturating small fragments of copal in a mortar with fine sand, pouring on chloroform to the powder thus obtained and filtering. The objects are brought into a capsule filled with the copal solution. The solution is now slowly evaporated by gently heating the capsule on a tile by means of a common night-light placed beneath it. As soon as the solution is so far concentrated as to draw out into threads that are brittle after cooling, the objects are removed from the capsule and placed to dry for a few days on the tile in order that they may more quickly become hard. When they have attained such a degree of hardness that they cannot be indented by a finger-nail, sections are cut from them by means of a fine saw. The sections are rubbed down even and smooth on one side with a hone, and cemented, with this side downwards, to a slide, by means either of Canada balsam or copal solution. The slide is put away for a few days more on the warmed tile. As soon as the cement is perfectly hard the sections are rubbed down on a grindstone, and then on a hone, to the requisite thinness and polish, washed with water, and mounted in balsam.

The process may be varied by imbedding the objects unstained, removing the copal from the sections by soaking in chloroform, decalcifying them if necessary, and then staining.

It is sometimes a good plan, after removing the copal, to cement a section to a slide by means of hard Canada balsam, then decalcify cautiously the exposed half of the specimen, wash, and stain it.

This method was invented in order to enable the hard and soft parts of corals to be studied in their natural relations, and is valuable for this and similar purposes.

178. Ehrenbaum's **Colophonium and Wax Method** (*Zeit. wiss. Mik.*, 1884, p. 414).—Ehrenbaum recommends a mass consisting of ten parts of colophonium to one of wax. The addition of wax makes the mass less brittle. Sections are obtained by grinding in the usual way. The mass is removed from them by means of turpentine followed by chloroform.

179. Johnstone-Lavis and Vosmaer's **Balsam Method** (*Journ. Roy. Mic. Soc.*, 1887, p. 200).—Alcohol material is carefully

and gradually saturated, first with benzol, and then with thin and thick solution of benzol-balsam. It is then dried for a day in the air and for several days more in a hot-air bath. When hard it is ground in the usual way.

180. **Weil's Canada Balsam Method,** see *Zeit. wiss. Mik.*, v, 1888, p. 200.

181. **Giesbrecht's Shellac Method.**—For hard parts only, spines of *Echinus*, shell, etc., see *Morph. Jahrb.*, vi, 1880, p. 95, or the abstract in Lee und Mayer, *Grundzüge*.

Congelation Masses.

182. **The Methods of Freezing.**—For the requisite manipulations, and means of producing the requisite degree of cold, see Carpenter's *The Microscope* (ether spray); Johne, *Zeit. wiss. Mik.*, xiv, 1897, p. 370 (liquid carbonic acid); Wolff, *ibid.*, xxv, 1908, p. 175 (ethyl chloride); Krause, *ibid.*, p. 289 (solid carbonic acid); Jung, *Verh. Ges. Naturf. Aertze*, lxix, 1898, p. 129 (ethyl chloride); Brissy, *C. R. Soc. Biol.*, lxii, 1907, p. 1115 (liquid air).

Fresh tissues may be, and are, frequently frozen *without being included in any mass.* But the *formation of ice crystals* frequently causes tearing of delicate elements, and it is better to *infiltrate the tissues* with a mass that does not crystallise in the freezing mixture, but becomes simply hard and tough, such as one of those given below.

When sections have been obtained, it is difficult to manipulate them. Olt (*Zeit. wiss. Mik.*, xxiii, 1906, p. 327) puts them into a 1 per cent. solution of gelatin, brings them therein on to a slide, hardens for an hour in vapour of formaldehyde, and soaks for a few minutes in formol of 10 per cent. Anitschkow (*ibid.*, xxvii, 1910, p. 73) puts them into alcohol of 50 per cent., gets them on to a slide prepared with Mayer's albumin, presses down with paper, puts into alcohol of 98 per cent., and thence through lower grades into water.

183. **Gum and Syrup Masses.**—Hamilton (*Journ. of Anat. and Phys.*, xii, 1878, p. 254) soaked tissues in syrup made with double refined sugar, 2 ounces; water, 1 fluid ounce;

then washed the superfluous syrup from the surface, and put into ordinary gum mucilage for an hour or so, and then imbedded in the freezing microtome with mucilage in the usual way.

Cole (*Methods of Microscopical Research*, 1884, p. xxxix) takes gum mucilage (B. P.), 5 parts; syrup, 3 parts. (For brain and spinal cord, retina, and all tissues liable to come in pieces put 4 parts of syrup to five of gum.) Add 5 grains of pure carbolic acid to each ounce of the medium.

(Gum mucilage [B. P.] is made by dissolving 4 ounces of picked gum acacia in 6 ounces of water. The syrup is made by dissolving 1 pound of loaf sugar in 1 pint of water and boiling.)

The freezing is conducted as follows:—The gum and syrup is removed from the *outside* of the object by means of a cloth; the spray is set going and a little gum mucilage painted on the freezing plate; the object is placed on this and surrounded with gum mucilage; it is thus saturated with gum and syrup, but surrounded when being frozen with mucilage only. This combination prevents the sections from curling up on the one hand, or splintering from being too hard frozen on the other. Should freezing have been carried too far, wait for a few seconds.

Webb (*The Microscope*, ix, 1890, p. 344; *Journ. Roy. Mic. Soc.*, 1890, p. 113) takes thick solution of dextrin in solution of carbolic acid in water (1 in 40).

184. Gelatin (Sollas, *Quart. Journ. Mic. Soc.*, xxiv, 1884, pp. 163, 164). **Gum Gelatin** (Jacobs, *Amer. Natural.*, 1885, p. 734). **White of Egg** (Rollett, *Denskschr. math. naturw. Kl. k. Acad. Wiss. Wien*, 1885; *Zeit. wiss. Mik.*, 1886, p. 92).—Small portions of tissue brought in the white of a freshly laid egg on to the freezing stage, frozen and cut. **Oil of Aniseed** (Kuhne, *Centralb. f. Bakteriol.*, xii, 1892, p. 28; *Journ. Roy. Micr. Soc.*, 1892, p. 706; V. A. Moore, *Amer. Mon. Mic. Journ.*, 1894, p. 373; *Journ. Roy. Mic. Soc.*, 1895, p. 247). **Anethol** (anise camphor), Stepanow, *Zeit. wiss. Mik.*, xvii, 1900, p. 181.

For details of these see previous editions.

For Döllken's method of solidifying formol by means of resorcin, see *Zeit. wiss. Mik.*, xiv, 1, 1897, p. 33.

CHAPTER X.

SERIAL SECTION MOUNTING.

185. Choice of a Method.—I recommend the following:—For *general* work with paraffin sections, *the combined water and albumen* method, § 188. For very delicate work, *the water method.* For collodion sections, the *albumen method*; for large collodion sections, GRAHAM KERR's seems the most convenient.

Methods for Paraffin Sections.

186. The Water or Dessication Method.—GAULE (*Arch. Anat. Phys., Phys. Abth.*, 1881, p. 156); SUCHANNEK (*Zeit. wiss. Mik.*, vii, 1891, p. 464); GULLAND (*Journ. Anat. and Phys.*, xxvi, 1891, p. 56); SCHIEFFERDECKER (*Zeit. wiss. Mik.*, ix, 1892, p. 202); HEIDENHAIN (*Kern und Protoplasma*, p. 114); NUSBAUM (*Anat. Anz.*, xii, 2, 1896, p. 52); MAYER in the *Grundzüge*, LEE und MAYER, 1898, p. 113; DE GROOT (*Zeit. wiss. Mik.*, xv, 1898, p. 62), and others.—The principle of this method is that the sections are made to adhere to the slide without the intervention of any cementing substance, being brought into intimate contact with the glass by being slowly drawn down by the evaporation of a layer of water on which they are floated. It is now practised, with unessential variations, as follows:

(*a*) For sections that are *large and not numerous.* The sections are flattened out on water by one or other of the processes described in § 149. The slide is then drained and put away to dry until every trace of water has completely evaporated away from under the sections. This drying may be performed at the temperature of the laboratory, in which case many hours will be necessary (to be safe it will generally be necessary to leave the sections overnight). Or it may be performed in a stove or on a water-bath at a temperature a few degrees *below* the melting-point of the paraffin (best not above 40° C.), in which case fixation will be much more rapid, large thin sections being often sufficiently fixed in an hour, though thick ones will require half a dozen hours or more. *The paraffin must not be allowed to melt before the sections are perfectly dry,* the sections are sure to become detached if it does. Perfectly dry sections have a certain brilliant transparent look that is easily recognisable. As soon as dry the paraffin may be removed and they may be further treated as desired. To remove the paraffin all that is requisite is to put the slide into a tube of xylol or other good solvent, which in a few seconds, or minutes at most, removes the paraffin perfectly. Most workers first melt the paraffin, but I find this is not necessary.

(*b*) For series of *numerous small sections.* Clean a slide perfectly, so that water will spread on it without any tendency to run into drops (see below). Breathe on it, and with a brush draw on it a streak of water as wide as the sections and a little longer than the first row of sections that it is intended to mount. With a dry brush arrange the first row of sections (which may be either loose ones or a length of a ribbon) on this streak. Breathe on the slide again, draw on it another streak of water under the first one and arrange the next row of sections on it, and so on until the slide is full. Then breathe on the slide again, and with the brush add a drop of water at each end of each row of sections, so as to enable them to expand freely; then warm the slide so as to flatten out the sections, taking care *not to melt the paraffin.* Some persons do this by holding it over a small flame for a few seconds. I prefer to lay it on a slab of thick glass, warmed, watching the flattening of the sections through a lens if necessary. As soon as they are perfectly flat, draw off the excess of water from one corner of the mount with a dry brush, and put aside to dry as before (*a*).

In order to succeed in this method it is absolutely essential that the sections be perfectly expanded and come into close contact with the slide at all points. And to ensure this it is necessary that the slide should be perfectly *free from grease,* so that the water may wet it equally everywhere. The test for this is, firstly, to breathe on the slide; the moisture from the breath should condense on it evenly all over, and disappear evenly. Secondly, streaks of water drawn on it with a brush should not run. To obtain a slide that will fulfil these conditions, clean it well in the usual way, place a drop of water on it and rub it in thoroughly with a damp cloth and try the tests. If this does not suffice, take a turn of a corner of the cloth round a finger and rub it with a piece of chalk, then damp the cloth and rub the slide with it, finishing up with a clean part of the cloth and clean water (DE GROOT, *loc. cit. supra*). If after performing this operation twice the slide still refuses to take the water thoroughly, it should be rejected as incorrigible; for there are apparently some sorts of glass that can never be got to wet properly. Mayer finds carbonate of magnesia or soda useful.

GUDERNATSCH (*Zeit. wiss. Mikr.*, xxiv, 1908, p. 358) washes the slide well with potash soap, and arranges the sections on it whilst still wet. HELLY (*ibid.*, 1906, p. 330) passes it two or three times over the flame of a Bunsen burner.

Tap water seems preferable to distilled water; it seems to spread better and give a stronger adhesion. NUSBAUM adds a trace of gum arabic (one or two drops of mucilage to a glass of water); APÁTHY (*Microtechnik*, p. 126) adds 1 per cent. of Mayer's albumen (§ 187); and HENNEGUY (*Leçons sur la Cellule*, 1896, p. 62) takes a 1 : 5000 solution of gelatin, with a trace of bichromate of potash, added just before using, and dries the slides exposed to light. Similarly, BURCHARDT (*Jena Zeit.*, xxxiv, 1900, p. 719).

Some workers have used alcohol (50 per cent. or 70 per cent.) instead of water; but this I believe to be now generally abandoned.

This is the most elegant method of any, as there is nothing on the slide except the sections that can stain, or appear as *dirt in the mount*. Tissues do not suffer from the drying, provided the material has been properly imbedded. Sections stick so fast by this method that they will stand watery or other fluids for weeks, *so long as they are not alkaline*. When successfully performed it is quite safe, provided that the sections are *of a suitable nature*. They must be such as to afford a sufficient *continuous surface*, everywhere in contact with the slide. Sections of parenchymatous organs stick well; sections of thin-walled tubular organs stick badly. Sections of chitinous organs are very unsafe. The larger and *thinner* sections are, the better do they stick, and *vice versâ*. Sections from chromic or osmic material adhere less well than sections from alcohol or sublimate material.

By taking a staining solution instead of pure water for expanding, the sections can be got to stain at the same time, and so be brought into balsam without passing through alcohol; see MAYER, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 320; SCHMORL, *Path.-hist. Untersuchungsmethoden*, 1897, p. 38; SMITH, *Journ. Anat. Phys.*, xxxiv, 1899, p. 151.

187. MAYER'S Albumen (*Mitth. Zool. Stat. Neapel*, iv, 1883; *Internat. Monatschr. f. Anat.*, iv, 1887, p. 42).—White of egg, 50 c.c.; glycerin, 50 c.c.; salicylate of soda, 1 grm. Shake them well together, and filter into a clean bottle. The filtering may take days or a week, but the preparation does not spoil meanwhile.

FRANCOTTE shakes up the albumen with a few drops of acetic acid before adding the other ingredients, and finds the filtering greatly quickened. So do I. Be careful with the acid.

A *very thin* layer of the mixture is spread on a slide with a fine brush and well rubbed in with the finger (I prefer a small rubber "squeegee"). The sections are laid on it and pressed down lightly with a brush (if they will bear it). The slide may then be warmed for some minutes on a water-bath, and the paraffin removed with a solvent.

It is *not necessary* to warm the slide at all; *the paraffin can be removed in the cold if desired* by putting the slide into toluol, xylol, or the like. But the slide *must*, in any case, be *treated with alcohol* after removal of the paraffin, in order to get rid of the glycerin, which will cause cloudiness if not perfectly removed.

This method allows of the staining of sections on the slide with perfect safety, both with alcoholic and aqueous stains, provided they be *not alkaline*.

According to my experience, the albumen method is *absolutely safe*, provided that *alkaline fluids be avoided* in the after-treatment. It has the defect that certain plasma stains (not chromatin stains) colour the albumen very strongly, and cannot be removed from it, and that sections are not expanded by it.

It sometimes happens that the mixture after it has stood for some time becomes turbid, and at last coagulates, passing into a caseous state; or it may undergo a hyaline coagulation, drying up like amber. But up to the very last it does not *in general* lose its adhesive properties. I have, however, found it to do so, after keeping for five or six years, so that, to be on the safe side, it may be well to make it up fresh every six months.

HEIDENHAIN (*Zeit. wiss. Mikr.*, xxii, 1905, p. 331) makes it up with 1 grm. of blood albumen dissolved in 25 c.c. of water, and an equal volume of 50 per cent. alcohol.

188. The Albumen and Water Method (HENNEGUY, *Journ. de l'Anat. et de la Physiol.*, 1891, p. 398).—A drop of water is spread on a slide painted with Mayer's white-of-egg mixture, the sections are arranged on it, the whole is warmed (*not* to the melting-point of the paraffin) until the sections flatten out; the water is then evaporated off at a temperature of about 40° C., and as soon as it has sufficiently disappeared, which at that temperature will be in about *ten to fifteen minutes*, the slide is further treated as described last §.

This is a most valuable method. It is quicker than the water method, and, for difficult material, safer.

See also OHLMACHER, *Journ. Amer. Med. Assoc.*, April, 1893.

The so-called "Japanese" method, attributed to IKEDA by REINKE (*Zeit. wiss. Mik.*, xii, 1895, p. 21), *is merely that of* HENNEGUY.

MANN (*Anat. Anz.*, viii, 1893, p. 442) shakes up white of egg with water, coats slides with it and dries them. He flattens sections on water at 40° C., lifts them out on a prepared slide, and dries for five minutes at 35° C.

189. Garlic-water.—HOLLANDE (*Arch. d'Anat. Micr.*, xiii, 1911, p. 171) gives the following as more adhesive than albumen:—50 g. of crushed and chopped garlic are rubbed up with 80 c.c. of chloroform-water (Codex, A.C.) and filtered after twenty-four hours. Use as albumen.

180. SCHÄLLIBAUM'S Collodion (*Arch. mikr. Anat.*, xxii, 1883, p. 565).—One part of collodion shaken up with 3–4 parts of clove or lavender oil. Use as albumen. Sections can be treated with alcohol (not absolute) and divers staining fluids. I do not find it safe for this. RABL, however (*Zeit. wiss. Mik.*, xi, 1894, p. 170), finds that it is if you take 2 parts of collodion to 3 of clove oil, and make up fresh every four or five days.

191. **OBREGIA'S Method for Paraffin or Celloidin Sections** (*Neurologisches Centralb.*, ix, 1890, p. 295; GULLAND, *Journ. of Path.*, February, 1893).—Slides, or glass plates of any size, are coated with a solution made of—

Syrupy solution of powdered candy-sugar made with boiling distilled water	30 c.c.
95 per cent. alcohol . . .	20 ,,
Transparent syrupy solution of pure dextrin made by boiling with distilled water	10 ,,

They are dried slowly for two or three days until the surface is just sticky to the moist finger. *Paraffin sections* are arranged and heated for a few minutes to a temperature slightly above the melting-point of the paraffin. The paraffin is removed by some solvent, and this in turn by absolute alcohol. The alcohol is poured off, and the sections are covered with solution of celloidin. The plates are left to evaporate for ten minutes in a horizontal position, then brought into water, in which the sheet of celloidin with the sections soon becomes detached, and may be further treated as desired, *e. g.* as in Weigert's process, § 198. The evaporation must not be artificially hastened.

DIMMER (*Zeit. wiss. Mik.*, xvi, 1899, p. 44) coats the slides with a solution of about 16 parts of gelatin in 300 of warm water, and dries them (two days), and proceeds in other respects as above.

A good method for *large* sections, equally applicable to paraffin sections, to celloidin sections, and to sections of material that has not been imbedded at all.

For BLOCHMAN'S modification of Weigert's process, by means of which large sections can be preserved *unmounted*, see *Zeit. wiss. Mik.*, xiv, 1897, p. 189.

192. **STRASSER'S Collodion Paper Method** (*ibid.*, iii, 1886, p. 346).—This is an extremely complicated modification of Weigert's method for celloidin sections, and is only adapted for use with STRASSER'S automatic ribbon-microtome. See *Zeit. wiss. Mik.*, iii, 1886, p. 346; vi, 1889, p. 154; vii, 1890, pp. 290 and 304; ix, 1892, p. 8; xii, 1895, p. 154; and xiv, 1897, p. 39; also SCHOENEMANN, *ibid.*, xix, 1903, p. 333; STRASSER, *ibid.*, p. 337; and RUPPRICHT, *ibid.*, xxviii, 1912, p. 281.

Methods for Watery Sections.

193. Fol's **Gelatin** (Fol, *Lehrb.*, p. 132).—Four grammes of gelatin are dissolved in 20 c.c. of glacial acetic acid by heating on a water-bath and agitation. To 5 c.c. of the solution add 70 c.c. of 70 per cent. alcohol and 1 to 2 c.c. of 5 per cent. aqueous solution of chrome-alum. Pour the mixture on to the slide and allow it to dry. In a few hours the gelatin passes into the insoluble state. It retains, however, the property of swelling and becoming somewhat sticky in presence of water. The slide may then be immersed in water containing the sections; these can be slid into their places, and the whole lifted out; the sections will be found to be fixed.

This method is specially intended for sections made under water, *large celloidin sections* amongst others.

Similarly, Ruppricht, *loc. cit.*, last §, with the needless complication of a seriation on Strasser's collodionised paper.

Strasser (*loc. cit.*, last §) also employs a dry gelatin film which he makes sticky by means of carbol-xylol.

Methods for Celloidin Sections.

194. **The Albumen Method.**—I find that celloidin sections may be mounted on Mayer's albumen, and have the celloidin removed, if desired, by putting them into ether-alcohol. Care must be taken to press them down very thoroughly on to the albumen; and it is well not to have them too wet.

Similarily Jordan (*Zeit. wiss. Mik.*, xv, 1898, p. 54), and Argutinsky, *ibid.* xvii, 1900, p. 37. See also Jordan (*ibid.*, 192-194); Dantschakoff, *ibid.*, xxv, 1908, p. 35; Maximow, *ibid.*, xxvi, 1909, p. 184; Anitschkow, *ibid.*, xxvii, 1910, p. 68; Weber, *ibid.*, xxix, 1912, p. 186; Rubaschkin, *Anat. Anz.*, xxxi, 1907, p. 30. Weber paints over the series on the albumen with a layer of thin collodion, and puts into alcohol of 50 per cent., then into a mixture of equal parts of chloroform and absolute alcohol. After staining, pure absolute alcohol must be avoided.

195. Summers' **Ether Method** (*Amer. Mon. Mic. Journ.*,1887, p. 73).—Place the sections in 95 per cent. alcohol for a

minute or two, arrange on the slide, and then pour over the sections sulphuric ether *vapour*, from a bottle partly full of liquid ether. The colloidin will immediately soften and become perfectly transparent. Place the slide in 80 per cent. alcohol, or even directly in 95 per cent. if desired. I have not myself found this method safe.

Instead of pouring the ether vapour over the slide, it may, of course, be treated with ether vapour *in a preparation glass or similar arrangement*, which I think preferable.

GAGE (*Proc. Amer. Soc. Mic.*, 1892, p. 82) advises that the slide be one that has been previously coated with a 0·5 per cent. solution of white of egg and dried; the collodion adheres much more strongly to an albuminised surface.

AUBURTIN (*Anat. Anz.*, xiii, 1897, p. 90) arranges on a clean slide, dehydrates the sections with blotting-paper and treatment with absolute alcohol, then drops on to them a mixture of alcohol and ether which dissolves out the celloidin from the sections, then allows the thin collodion thus formed to evaporate into a thin sheet on the slide. Then 70 per cent. alcohol and other desired reagents.

Similarly, MAIER (*Münch. med. Wochenschr.*, lvii, 1910, No. 12; *Zeit. wiss. Mik.*, xxvii, 1910, p. 385), but adding a treatment for ten to fifteen minutes with sulphide of carbon.

See also MYERS, *Arch. Anat. Phys., Anat. Abth.*, 1902, p. 371 (complicated).

196. APÁTHY'S **Oil of Bergamot Method** (*Mitth. Zool. Stat. Neapel*, 1887, p. 742; *Zeit. wiss. Mik.*, v, 1888, pp. 46 and 360, and vi, 1889, p. 167).—Cut with a knife smeared with yellow vaseline and wetted with 95 per cent. alcohol. Float the sections, as cut, on bergamot oil (must be green, must mix perfectly with 90 per cent. alcohol, and must not smell of turpentine), or on carbolxyol (*Mikrotechnik*, p. 176). The sections *flatten themselves out* on the surface of the oil; and are then transferred to a slide which (APÁTHY, *Mikrotechnik*, pp. 127 and 176) has been previously collodionised and dried.

If the sections are to be stained, the slide after removal of the bergamot oil, by a cigarette paper, is exposed for a few minutes to the vapour of a mixture of ether and alcohol, then brought into 90 per cent. alcohol, and after a quarter of an hour therein may be stained in any fluid that contains 70 per cent. alcohol or more.

If it be desired to stain in a watery fluid, care must have been taken when arranging the sections to let the colloidin of each section overlap that of its neighbours at the edges, so that the ether vapour may fuse them all into one continuous plate. This will become detached from the slide in watery fluids, and may then be treated as a single section. Terpinol may be taken instead of bergamot oil.

197. Apáthy's **Series-on-the-Knife Method** (*Zeit. wiss. Mik.*, vi, 1888, p. 168).—The knife is well smeared with yellow vaseline, rubbed evenly on, and is wetted with alcohol of 70 to 90 per cent. As fast as the sections are cut they are drawn with a needle or small brush to a dry part of the blade, and there arranged in rows, the celloidin of each section everlapping or at least touching that of its neighbours. When a series (or several series, if you like) has been thus completed, the sections are dried by laying blotting-paper on them, and the series is painted over with some of the thinnest celloidin solution used for imbedding, is allowed to evaporate for five minutes in the air, and the knife is then removed and brought for half an hour into 70 per cent. alcohol. This hardens the celloidin around the sections into a continuous lamella, which can be easily detached by means of a scalpel, and stained, or further treated as desired.

198. Weigért's Collodion Method (*Zeit. wiss. Mikr.*, 1885, p. 490).—Slides, or larger plates of glass, are prepared by coating them with collodion in a thin layer, as photographers do, and allowing them to dry (they may be kept thus in stock). Sections (cut wet with alcohol) are got on to one of these (by a roundabout process, not essential), and arranged in order, and gently pressed down with paper.

Now remove with blotting-paper any excess of alcohol that may remain on or around the sections, pour collodion over them, and get it to spread in an even layer. As soon as this layer is dry at the surface you may write any necessary indications on it with a small brush charged with methylen blue (the colour will remain fast throughout all subsequent manipulations).

The plate may now be either put away till wanted in 80 per cent. alcohol, or may be brought into a staining fluid.

The watery fluid causes the double sheet of collodion to become detached from the glass, holding the sections fast between its folds. It is then easy to stain, wash, dehydrate, and mount in the usual way, merely taking care not to use alcohol of more than 90 to 96 per cent. for dehydration. Weigert recommends for clearing the mixture of xylol and carbolic acid (§ 167).

The series should be cut into the desired lengths for mounting whilst in the alcohol.

A good method for *large* and *thick* sections.

For Blochman's modification see § 191.

Strasser takes gummed paper instead of the glass plates used in this process. See the papers quoted § 192.

See also Wintersteiner (*Zeit. wiss. Mik.*, x, 1893, p. 316) and Kubo (*Arch. mik. Anat.*, lxx, 1907, p. 173).

199. Obregia's Method.—Slides are prepared as directed (§ 191), the sections are arranged on them and covered with celloidin or photoxylin and evaporated as described, § 191.

For Dimmer's modification see also § 191.

200. Collodion Film Method.—Graham Kerr (*in litt.*, 1908) seriates on *Kodak films*. A film has the emulsion removed by hot water. The sections are arranged on a dry film, and the applications of a drop of absolute alcohol and ether (or an atmosphere of alcohol and ether) suffices to weld them into a mass with the film. The sheet may then be stained and mounted, or rolled up and stored in cedar oil.

Other Methods for Celloidin Sections.—See §§ 193 (Fol) and 182 (Olt).

CHAPTER XI.

STAINING.

201. The Molecular Processes involved in Staining.—The question whether the phenomena of staining and of industrial dyeing are chiefly of a chemical order, as held by some, or chiefly of a physical order, as held by others, is outside the province of this book. See (besides works on chemistry, amongst which may be mentioned Benedikt and Knecht, *The Chemistry of the Coal-tar Colours*, London, 1889) Fischer's *Fixirung, Färbung und Bau des Protoplasmus*, Jena, G. Fischer, 1899; Pappenheim's *Grundriss der Farbchemie*, Berlin, A. Hirschwald, 1901; and the articles in *Encycl. mik. Technik.*

202. Histological Staining: Specific, Nuclear, and Plasmatic.—Stains are either *general* or *special* (otherwise called Specific, or Selective, or Elective). A general stain is one that takes effect on all the elements of a preparation. A special, specific, selective, or elective stain is one that takes effect only on some of them, certain elements being made prominent by being coloured, the rest either remaining colourless or being coloured with a different intensity or in a different tone. To obtain this *differentiation* is the chief object for which colouring reagents are employed in microscopic anatomy.

Two chief kinds of this selection may be distinguished—*histological* selection and *cytological* selection. In the former an entire tissue or group of tissue elements is prominently stained, the elements of other sorts present in the preparation remaining colourless or being at all events differently stained, as in a successful impregnation of nerve-endings by means of gold chloride. This is the kind of stain that is generally meant by a *specific* stain. In the latter the stain seizes on one of the constituent elements of cells in general, for instance, either on the chromatin of the nucleus, or on one or other of the elements that go to make up the cytoplasm.

Stains that thus exhibit a selective affinity for the substance of nuclei—*nuclear* or *chromatin* stains—form a class of stains of peculiar importance for the embryologist or zootomist. For they enable him to have the nuclei of tissues marked out by staining in the midst of the unstained material in such a way that they may form landmarks to catch the eye, which is then able to follow out with ease the contours and relations of the elements to which the nuclei belong.

To these must be added another group of stains of the greatest importance to the cytologist and histologist, the *plasmatic stains* or *plasma* stains. These take effect especially on elements of cells and tissues other than the chromatin—for instance, on the reticulum of cytoplasm, or on its granules, or on polar corpuscles, etc., or on the formed material of tissues.

203. Dyes: Basic, Acid, and Neutral.—The colouring matters, or dyes, employed either in industrial dyeing or in histological staining are almost always *salts*. They are known as "*basic*," "*acid*," or "*neutral*" dyes. By a "basic" dye is meant one which is either a base or a compound in which a so-called "colour base" (or molecular group to which the compound owes its colouring properties) is combined with a non-colouring acid. For instance, fuchsin or magenta is a basic dye. It is the hydrochloride of rosanilin, and its colouring properties are due to the rosanilin which exists as a base in the compound, and not to the hydrochloric acid of the compound. By an "acid" dye is meant a compound in which a so-called "colour-acid" is combined with a non-colouring base. The dye known as acid fuchsin or acid magenta (Säurefuchsin) is an "acid" colour. It is the soda-salt of di- or tri-sulphoconjugated rosanilin, that is of rosanilin di- or tri-sulphonic acid, and its colouring properties are due to the rosanilin which exists as an acid in the compound, and not to the soda. Or, to take a simpler case, picrate of ammonia is an "acid" colour, and its colouring properties are evidently due to the picric acid in it, and not to the ammonia.

It is important to keep clearly in mind that in speaking of dyes the terms "acid" or "basic" refer to the characters of the colour acids or colour bases, and not to those of the salts. An "acid" dye may have a neutral or alkaline reaction (*e. g.* picrate of ammonia), and *vice versâ.*

Basic dyes are generally easily soluble in alcohol, less easily in water; whilst the contrary is the case for acid dyes. The free colour bases or colour-acids are generally less soluble in water than their salts, for which reason they are so little used that they are hardly to be found in commerce. It follows that such histological formulæ as depend on setting free a colour-acid from its salt (*e.g.* as by precipitating it from eosin by means of alum, as advised by Ranvier and Wissotzky) are irrational. Colour-bases or colour-acids may themselves be colourless.

The stain given by acid dyes is fast to acids, and may be intensified by them; whilst basic dyes are washed out by acids, but intensified by alkalies.

"Neutral" dyes are compounds of a colour-base with a colour-acid. They are seldom or never prepared industrially, the only example that I can find mentioned in Benedikt and Knecht's *Chemistry of the Coal-tar Colours* being artificial indigo. They are prepared for histological purposes by mixing the aqueous solutions of a basic and an acid dye. For instance, by mixing the acid picrate of ammonia with the basic hydrochloride of rosanilin, you can bring about the formation of sal ammoniac and picrate of rosanilin, which is a "neutral" colouring matter. They are generally insoluble in pure water, and hence precipitate when the mixture is made, but may be got to redissolve by adding an excess of the acid colour, or of the basic, and are always soluble in alcohol. They can also, as is often done, be formed in the tissues themselves by staining first with an acid dye, and then bringing the preparation, without first washing out, into a basic dye.

Ehrlich and Lazarus ("Die Anæmie," Wien, 1898, p. 26) state that the basic dyes methyl-green, methylen-blue, amethyst violet (also pyronin and rhodamin), and the acid dyes Säurefuchsin, Orange G, and Narceïn, are peculiarly favourable for making neutral mixtures.

See further as to the "neutral" colours, Rosin, "Ueber eine neue Gruppe der Anilinfarbstoffen," in *Berliner klin. Wochenschr.*, xii, 1898,

p. 251; *Zeit. f. wiss. Mik.*, xvi, 2, 1899, p. 223; *Journ. Roy. Mic. Soc.*, 1899, p. 547; *Encycl. mik. Technik*, 1903, p. 1028; PAPPENHEIM, *op. cit. supra*; HEIDENHAIN, *Anat. Anz.*, xx, 1901, p. 36.

204. **The Chromatophily of Tissue-Elements.**—The elements of tissues are distinguished as "basophilous," "acidophilous," and "neutrophilous," according as they seem to show a natural affinity for basic, acid, or neutral colouring matters respectively. According to a generalisation due to EHRLICH (*Zeit. klin. Med.*, 1, 1880, p. 555; REICHERT AND DU-BOIS REYMOND'S *Arch. Anat. Phys., Phys. Abth.*, 1879, p. 571), the basic colours are in general chromatin stains—that is, they have a special affinity for the element of nuclei known as chromatin, so that they are mostly sharp nuclear stains, and chromatin is *basophilous*. The acid colours, on the other hand, are, according to him, in general plasma stains—that is, they have a special affinity for cytoplasm and intercellular substances, which are therefore *acidophilous*. The neutral colours exhibit special affinities for certain cell-contents, and the elements affected by these are said to be *neutrophilous*.

I think that that is a generalisation which requires some explanation and qualification. In practical histology we have to take account not only of the affinities for dyes of cellular elements in a physically and chemically unaltered state, but of the alterations in these affinities brought about by the action of fixatives and mordants. Now most fixing agents either diminish or increase the chromatophily of tissues; so also do all mordants, some of which may even invert the natural chromatophily of tissues (see §§ 205, 207). Then, too, we have to take account also of the resistance of the stain to the liquids employed for washing, for dehydration, for clearing; in short, we have to take into account the way in which the dye behaves when employed as a *regressive* stain (§ 209). This is of peculiar importance in the case of the coal-tar colours, seeing that they are largely used for the *regressive* staining of sections destined to be dehydrated by alcohol and mounted in balsam. Now Ehrlich's experiments take no account of these conditions. (He worked with "cover-glass preparations" of isolated cells, such as blood and lymph cells, and was thus able to avoid the prolonged washing necessary for most sections, and to suppress altogether the dehydration by alcohol, his cover-glass preparations being simply dried after staining in a stove.) In consequence, his chemical categories of *basic colours* and *acid colours* fail to correspond always *in practice* to the technical categories of *chromatin stains* and *plasma stains*.

For instance, orange is an acid colour; but used as a regressive stain I find it will give a very sharp stain of chromatin: it cannot, therefore, be classed as a mere plasma stain, though it is also a very good plasma stain. Säurefuchsin is a very acid colour. It behaves in general as a decided plasma stain. But used as a regressive stain it sometimes, under conditions which I am not able to specify, gives a very vigorous stain of chromatin. Safranin is a basic colour, but by the use of appropriate mordants it can be made to behave as a plasma stain. Methylen blue is a basic colour. But, as is well known, when employed according to the method worked out by Ehrlich for the so-called *intra-vitam* staining of nerves, it affords a stain that is essentially plasmatic, such staining of nuclei as may occur in this process being an accidental epiphenomenon. Nigrosin is, according to Ehrlich, an acid colour, and should therefore be essentially a plasma stain. Yet I find that, used as a regressive stain in the same way as safranin, it gives a vigorous chromatin stain, cytoplasm being only faintly coloured. Bordeaux is an acid colour, but it stains chromatin as well as cytoplasm. Further, both carminic acid and hæmatein are acid dyes, but combined with the mordant, alum (as in alum-carmine or alum-hæmatoxylin), they give nuclear stains. Indeed, it is not too much to assert that there is hardly any colour, either basic or acid, that may not be made to afford either a chromatin stain or a plasma stain, according to the way in which it is employed. There is, in practice, no *absolute* chromatophily of tissue-elements.

205. Substantive and Adjective Staining; Mordants.—In the industry of dyeing, colouring matters are divided into two classes, according to their behaviour with respect to the material to be dyed. Certain dyes are absorbed directly from their solutions by the material immersed therein, and combine with it directly. In this case the material is said to be *substantively* dyed, and the colouring matter is called a *substantive* colouring matter.

Other dyes do not combine directly with the material to be acted on, but this material must first be charged with some substance known as a *mordant* (generally a metallic salt or hydrate) before it will combine with the colouring matter. These are known as *adjective* colouring matters.*

Mordants are bodies which have the property of combining on the one hand with the elements of tissues and on the other with the colouring principle of the dyes used, forming with the latter insoluble coloured compounds (known as Lakes), which are retained in the tissues. It follows that basic dyes require mordants of an acid character and acid dyes mordants of a basic character. The mordant may be introduced into the tissues either before the dye or at the same time with it.

It may seem at first sight that the majority of histological stains are obtained by substantive staining of the tissues. But on reflection it will be seen that many of the histological stains that are obtained without intentional mordanting of the tissues should yet in strictness be attributed to the class of adjective stains. For whenever there is reason to suppose that the stain obtained results from a combination of the colouring matter with some metallic salt or hydrate that is not a constituent of the living tissue, but has been brought into it by the fixing or hardening reagents, it must be admitted that these reagents play the part of mordants though only intentionally employed for another purpose. This would appear to be the case with the stains, or some of them, obtained after fixation with corrosive sublimate, picric acid, salts of iron, of platinum, of palladium, of uranium, and, for certain tissue elements and certain colours, chromium. And further, the mordanting substance may not only be present unintentionally in the fixing or hardening agents, it may be present unintentionally, or with imperfect realisation of its import, in the staining solutions themselves. Such is undoubtedly the part played by alum in most of the stains in which it figures as an ingredient. Iodine also plays in some staining processes a part which seems only explicable on the supposition that it acts as a mordant. And in some processes an acid dye is made to act as a mordant for a subsequently employed basic dye.

---

* For an excellent popular exposition of this subject see BENEDIKT and KNECHT's *Chemistry of the Coal-tar Colours* (George Bell and Sons).

In some staining processes, however, mordants are *intentionally* resorted to in order to fix the stain. Mordanting has long been intentionally employed in some hæmatein staining processes, such as the iron-alum process of BENDA and M. HEIDENHAIN. More lately it has been resorted to for staining with tar colours.

By combining with the elements of tissues, mordants confer on them an *artificial chromatophily*. This may take place to so great an extent that the original affinities of the tissues for dyes are not only masked but may be seemingly altered into their opposites, thus producing an "inversion" of their chromatophily. Thus by means of appropriate acid mordants certain basic anilins, which by the usual methods of regressive staining are pure chromatin stains, may be made to afford a pure plasma stain—one not affecting chromatin at all, thus giving an "inversion" of the usual stain.

It would seem that the nuclear stains obtained by carmine and hæmatoxylin should in strictness be classed as inversion stains. The colouring principle of carmine, carminic acid, is an acid body, and consequently a plasma stain. But in the form of carmine it is combined with the basic oxide alumina, which by mordanting the acid body nuclein confers on it a basic character and renders it acidophilous, thus producing an inversion of the natural staining affinity. So also with the acid dye hæmatein. So that we see that whilst basic dyes substantively employed are nuclear stains and acid dyes substantively employed are plasma stains, yet basic colours with acid mordants may give a plasma stain and acid colours with basic mordants may give a nuclear stain.

206. Metachromasy.—Adjective stains are of the colour of the lake formed by the mordant and the colouring principle of the dye. Substantive stains are for the most part of the colour of the solution of the dye employed. But this is not always the case. There is a very small group of dyes, mostly basic tar-colours, which have the property of staining different elements of tissues in different colours, one of these being the same as that of the solution of the dye, and the other, or metachromatic colour, being the same as that of the free colour-base of the dye. For instance, the red dye, safranin, colours nuclei red, but mucin and the ground-substance of cartilage orange. The blue dye, methyl violet, stains normally blue; but amyloid matter, Mastzellen, mucin, and cartilage, are stained red by it. Similarly with thionin, dahlia, gentian violet, toluidin blue, etc.

In these cases the effect appears to be due to chemical reaction. But in other cases a similar effect has been shown to be due to the presence of impurities in the dyes; so with iodine green and methyl green, which mostly contain an admixture of methyl violet.

It has been held by some that metachromasy is an optical phenomenon, but that is clearly only true of certain cases.

207. The Preparation of Tissues for Staining.—It is *generally* found that precise stains can only be obtained with carefully *fixed* (*i. e.* hardened) tissues. Dead, but not artificially hardened tissues stain indeed, but not generally in a precise manner. Living tissue elements in general do not stain at all, but resist the action of colouring reagents till they are killed by them (see, however, next section).

It appears probable, as was first pointed out, I believe, by MAYER (*Mitth. Zool. Stat. Neapel*, ii, 1880, p. 17), that most of the histological stains obtained with fixed tissues are brought about in one of two ways. Either they result from the combination of the colouring agent with certain organic or inorganic salts,—phosphates, for instance, that existed in the tissue elements during life and were thrown down *in situ* by the fixing or hardening agent employed, as seems to happen when such a fixing agent as alcohol is employed. Or they result from the combination of the colouring agent with certain compounds that did not pre-exist in the tissues, but were formed by the combination of the constituents of the tissues with the chemical elements brought to them by the fixing agent, as seems to happen when such a fixing agent as chromic acid is employed—the compounds in question being probably chiefly metal albuminates. It follows that *correct fixation* and careful *washing out* are essential to the production of good stains.

Objects which have been passed through alcohol *generally* stain better than those which have only been in watery fluids.

But *long* preservation of tissues in alcohol is generally unfavourable to staining.

208. Staining "intra vitam."—Some few substances (which are almost always *basic* dyes) possess the property of staining —or rather, *tingeing*—living cells without greatly impairing their vitality. Such are—in very dilute solutions—cyanin (or quinoleïn), methylen blue, Bismarck brown, anilin black, Congo red, neutral red, Nile blue, Janus green, and, under certain conditions, dahlia and eosin, gentian violet, with perhaps methyl violet, and some others.

These reagents are best employed in a state of considerable dilution, and in neutral or feebly alkaline solution—acids being of course toxic to cells. Thus employed, they will be found to tinge with colour the *cytoplasm* of certain cells during life; *never*, so far as I can see, nuclear *chromatin* during life; if this stain, it is a sign that death has set in. The stain is sometimes diffused throughout the general substance of the cytoplasm, sometimes limited to certain granules in it.

It has been asserted by some observers that the nucleus may be stained during the life of the cell by means of Bismarck brown, Congo red, methylen blue, neutral red, Nile blue, and safranin. But it is by no means clear from the statements of these writers that the coloration observed by them is localised *in the chromatin* of the nucleus. It would rather appear to be a diffuse coloration brought about by mechanical and momentary retention of the dye in the nucleus—which is a very different thing from a true nuclear stain. And in some of the cases reported it is by no means certain that the coloured nuclei were really in the living state. See hereon the article by Fischel ("Färbungen, intravitale") in *Encycl. mik. Tecknik.*

I have myself made a considerable number of observasions on the subject of *intra-vitam* staining, and have come to the same conclusion as Galeotti (*Zeit. wiss. Mik.*, xi, 1894, p. 172), and many recent writers, namely, that most of the so-called "intra-vitam" stains are either not true stains or that the stained substances are not really living. The coloration appears mostly, if the cell that shows it has remained in a state of unimpaired vitality, to be due to simple absorption or imbibition of the colouring matter by the cell, not to a molecular combination of the colouring matter with any of the constituents of the cells.

And when a more or less fast stain has been obtained, it is generally found that this is limited to cell-contents that do not appear to form an integral part of the living texture of the cell—to food-granules, or katabolic products, or the like.

Fischel (*Anat. Hefte*, xvi, 1901, p. 417, and *op. cit. supra*) concludes in favour of the vitality of *certain* of the stained granules.

Apart, however, from this question, it must be conceded that these so-called "vital stains" are frequently very useful. According to my experience, methylen blue is the most generally useful of them. It has (with Bismarck brown, Congo red, and neutral red) the valuable point that it is sufficiently soluble in saline solutions, and may therefore be employed with marine organisms by simply adding it to sea-water. The others are not thus soluble to a practical extent, but I find that gentian and dahlia become so if a trace of chloral hydrate—0·25 per cent. is ample enough—be added to the saline solution. Any of these reagents may be rubbed up with serum, or other "indifferent" liquid.

Methylen blue may be fixed in the tissues, and permanent preparations made, by one or other of the methods described in Chap. XVI. Bismarck brown stains may be fixed with 0·2 per cent. chromic acid or with sublimate solution (Mayer), or 1 per cent. osmic acid (Loisel, *Journ. de l'Anat. et de la Phys.*, 1898, No. 2, p. 212—a work that contains a good deal of information on the subject of *intra-vitam* stains), and the preparations may be stained with safranin, care being taken not to expose them too long to the action of alcohol. For the study of cell-granules, neutral red is perhaps the best.

Fischel ("Unters. ueb. vitale Faerbungen," Leipzig. 1908) finds that alizarin is specific for nerves. Add excess of alizarin to boiling water, boil and filter, and add 1 vol. of the filtrate to the water containing the organisms (Cladocera). The stain takes several hours.

For sulphorhodamin, which is selective for many organs (kidney, liver, uterus, skin, lymph-glands, etc.), see Andreew, in *Virchow's Arch.*, cciv, 1911, p. 447.

See also Gollman, *Proc. Roy. Soc.*, lxxxv, 1912, p. 146 (trypan blue, isamin blue, diamin blue, etc.); and Goldmann, "Die aeussere u. innere Sekretion, etc.," Tübingen, 1909, and "Neue Untersuch., etc.," ibid., 1912.

209. The Practice of Staining.—Selective staining is arrived at in two ways. In the one, which is called the *progressive* method, you make use of a colouring reagent that stains the elements desired to be selected more quickly than the elements you wish to have unstained; and you stop the process and fix the colour at the moment when the former are just sufficiently stained, and the latter not affected to an injurious extent, or not affected at all, by the colour. This is what happens, for instance, when you stain the nuclei of a preparation by treatment with very dilute alum hæmatoxylin: you get, at a certain moment, a fairly pure nuclear stain; but if you were to prolong the treatment, the extra-nuclear elements would take up the colour, and the selectivity of the stain would be lost. This is in general the method employed for the colouring of specimens *in bulk*—a procedure which is not possible with most of the regressive strains. It is the old method of carmine and hæmatoxylin staining.

The second, or *regressive* method, is the method of over-staining followed by partial decoloration. You begin by staining all the elements of your preparation indiscriminately, and you then wash out the colour from all the elements except those which you desire to have stained, these retaining the colour more obstinately than the others, in virtue of their chemical or physical constitution. This is what happens, for instance, when you stain a section of one deep red in all its elements with safranin, and then, treating for a few seconds with alcohol, extract the colour from all but the chromatin and nucleoli of the nuclei. This method is in general applicable *only to sections*, and not to staining objects *in bulk* (the case of borax carmine, with a few others, is an exception). It is a method, however, of very wide applicability, and gives, perhaps, the most brilliant results that have hitherto been attained. It frequently enables us to obtain a powerful stain of certain elements that would not be sufficiently brought out by the progressive method.

Tissues are stained either in *bulk* or in *sections*. For accurate work, such as is necessary in cytology and frequently in histology, it is greatly preferable, sometimes even necessary, to stain the sections, as by this means only is accurate control of the staining process under the microscope possible.

Staining solutions are mostly made with either *water* or *alcohol* as a menstruum. Water is generally preferable so far as the quality of the stain is concerned; but alcohol is frequently indicated, both on account of its greater power of penetration and as being less injurious to tissues. It is a good rule not to let staining baths contain more than fifty per cent. of alcohol.

Better results (as regards the quality of the stain, not as regards the preservation of the tissues) are generally obtained by *prolonged* staining in very dilute solutions, rather than by a *short* bath in a strong one.

210. Choice of a Stain.—The following may be recommended to the *beginner* for general work:—*For sections*, Mayer's *hæmalum*; or, for chromosmium objects more especially, Benda's or Heidenhain's iron hæmatoxylin.

For staining *in toto* Grenacher's alcoholic *borax-carmine*, or Mayer's *carmalum*, or hæmalum, unless the object be so impermeable as to require a very highly alcoholised stain, in which case take Mayer's *paracarmine*, or for chromic acid objects Mayer's *hæmacalcium*.

For *fresh tissues* or small entire objects, *methyl green*, if it is not important to have permanent preparations; if it is, take *carmalum* or *alum-carmine* (but both of these may give precipitates with marine animals).

Most of the carmine and hæmatoxylin solutions, properly used, give stains that are indefinitely permanent—at least in balsam. But most of the stains obtained with coal-tar dyes fade much in a few months or years. The most permanent are safranin, gentian violet, Bismark brown, and picric acid, which fade very little. Victoria blue, I find, is also fairly permanent.

CHAPTER XII.

CARMINE AND COCHINEAL STAINS.

211. **Carmine.**—Carmine is by no means merely carminic acid with at most certain impurities. According to the analysis of LIEBERMANN (*Ber. d. Chem. Ges.*, Jahrg. 18, 1886, pp. 1969—1975) it is a *very peculiar alumina-lime-protein compound of carminic acid*, a true chemical compound from which at all events *aluminium* and *calcium* can no more be absent than sodium from salt. It results from the researches of MAYER (*Mitth. Zool. Stat. Neapel.*, x, 1892, p. 480) that in the processes of histological staining (*not* of industrial dyeing) the active factors of the compound are, besides the carminic acid, always the *alumina*, and in some cases the lime. *The other bases are inactive*; the nitrogenous matters, so far as they have any influence at all, are an obstacle, as it is they that give rise to the well-known putrefaction of the solutions.

This being so, it follows that *carminic acid* may, if desired, *be taken as the basis of staining solutions instead of carmine.* Staining solutions thus prepared do not give essentially better stains than those made with carmine; but have the advantage of being of more constant composition. For carmine is a product which varies greatly from sample to sample.

Carminic acid of sufficient purity is furnished by GRÜBLER and HOLLBORN (or C. A. F. KAHLBAUM, in Berlin). It is soluble in water and *weak* alcohol (that of 70 per cent. only dissolves less than 3 per cent.) It cannot be used *alone* for staining, as it only gives in this way a weak and diffuse stain.

212. **Cochineal.**—According to MAYER (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 496), the active principle of extract or tincture of cochineal (as used in histology) is not free carminic acid, but carminic acid chemically combined with a base which is not lime, but some alkali. The watery extract

made with *alum*, or cochineal-alum carmine (§ 216), owes its staining-power to the formation of carminate of alumina (last §). The tincture made with *pure alcohol*, on the other hand, contains only the above-mentioned carminate of some alkali. This carminate *alone* stains weakly and diffusely (like carminic acid alone). But if in the tissues treated with it it meet with lime salts, alumina or magnesia salts, or even metallic salts capable of combining with it and forming insoluble coloured precipitates in the tissues, then a strong and selective stain may result. And if the necessary salts be added to the tincture itself, there results a solution containing the necessary elements for affording a strong and selective stain with all classes of objects. Hence Mayer's new formula, § 236.

213. General Remarks.—Carmine stains are chiefly used for staining *entire objects*, or tissues in bulk. In most cases this can be done more satisfactorily by means of carmine than by means of any other known agent. For most hæmateïn solutions have a disastrous tendency to overstain; and the tar-colours are generally inapplicable to staining in bulk.

Grenacher's alcoholic borax-carmine may be recommended to the beginner as being the easiest of these stains to work with: or para-carmine, for objects which require a highly alcoholic solution. Carmalum, or one of the alum-carmines, is also an easy and safe reagent.

Overstains may in all cases be washed out with weak HCl (*e.g.* 0·1 per cent.). Alum-solution will often suffice, or, according to Henneguy (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 400), permanganate of potash. All carmine stains, with the exception of aceto-carmine, are permanent in balsam. The alum-carmines are fairly permanent in glycerin. None of the acid stains, nor any of Grenacher's fluids, should be used with calcareous structures that it is wished to preserve, unless they be taken in a state of extreme dilution.

A. Aqueous Carmine Stains.

a. Acid.

214. Alum-carmine (Grenacher, *Arch. mik. Anat.*, xvi, 1879, p. 465).—An aqueous solution (of 1 to 5 per cent. strength, or any other strength that may be preferred) of common or ammonia alum is boiled for ten or twenty minutes with ½ to 1 per cent. of powdered carmine. (It is perhaps the safer plan to take the alum solution highly concentrated in the first instance, and after boiling the carmine in it dilute to the desired strength.) When cool, filter.

This stain must be avoided in the case of calcareous structures that it is wished to preserve.

TIZZONI (*Bull. Sc. Med. Bologna*, 1884, p. 259), PISENTI (*Gazz. degli Ospetali*, No. 24; *Zeit. wiss. Mik.*, ii, 1885, p. 378), and GRIEB (*Mem. Soc. Ital. Sci.*, t. vi, No. 9, 1887; *Zeit. wiss. Mik.*, vii, 1, 1890, p. 47) have given modifications of Grenacher's formula which do not appear to me rational.

MAYER (*ibid.*, xiv, 1897, p. 29) makes a stronger stain by taking 2 grms. carmine, 5 grms. alum, and 100 c.c. water, and boiling for an hour.

Alum-carmine is an *excellent* stain. It is particularly to be recommended to the beginner, as it is easy to work with; it is hardly possible to overstain with it. Its chief defect is that it is *not very penetrating*, and therefore unsuitable for staining objects of considerable size in bulk.

215. Acetic Acid Alum-Carmine (HENNEGUY, in *Traité des Méth. techn.*, LEE et HENNEGUY, 1887, p. 88).—Excess of carmine is boiled in saturated solution of potash alum. After cooling add 10 per cent. of glacial acetic acid, and leave to settle for some days, then filter.

For staining, enough of the solution is added to distilled water to give it a deep rose tint. In order to ensure rapid diffusion it is well to bring the tissues into the stain direct from alcohol. Stain for twenty-four to forty-eight hours, and wash for an hour or two in *distilled* water. Mount in balsam. You can mount in glycerin, but the preparations do not keep so well.

The advantage of this carmine is that it has much *greater power of penetration* than the non-acidified alum-carmine.

216. Cochineal Alum-carmine (PARTSCH, *Arch. mik. Anat.*, xiv, 1877, p. 180).—Powdered cochineal is boiled for some time in a 5 per cent. solution of alum, the decoction filtered, and a little salicylic acid added to preserve it from mould.

Another method of preparation has been given by CZOKOR (*ibid.*, xviii, 1880, p. 413).—Mayer finds that Partsch's is the more rational, the proportion of alum in it being exactly right, whilst in Czokor's it is insufficient. Partsch's fluid also keeps better.

Rabl (*Zeit. wiss. Mik.*, xi, 2, 1894, p. 168) takes 25 grms. each of cochineal and alum, 800 c.c. of water, and boils down to 600 c.c. He prefers this because it is *not so purely nuclear* a stain as the others.

These solutions give a stain that is practically identical with that of alum-carmine made from carmine, with perhaps even more delicate differentiations.

Rawitz (*Zeit. wiss. Mik.*, xxv, 1909, p. 392) takes cochineal 4 grms., nitrate of aluminium (or ammonio-sulphate of cobalt) 4 grms., water 100 c.c., and glycerin 100 c.c. Only for sections.

217. Mayer's **Carmalum** (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 489).—Carminic acid, 1 grm.; alum, 10 grms.; distilled water, 200 c.c. Dissolve with heat (if necessary). Decant or filter. Add some antiseptic, either 1 c.c. formol, or 0·1 per cent. salicylic acid, or 0·5 per cent. salicylate of soda. The solution will then keep. It *stains well in bulk*, even osmium objects. If washed out with distilled water only, the plasma will remain somewhat stained. If this be not desired, wash out carefully with alum solution, or, in difficult cases with weak acid, followed in either case with water. The general effect is that of an alum-carmine stain.

A weaker solution may be made by taking from three to five times as much alum and five times as much water, and dissolving in the cold.

With either solution the objects to be stained should *not* have an *alkaline reaction*.

Rawitz (*Anat. Anz.*, xv, 1899, p. 438) takes 2 grms. carminic acid, 20 grms. ammonia-alum, 150 c.c. water, and 150 c.c. glycerin. A strongly staining solution, which is said to *keep well*. Only for sections.

All solutions prepared with alum tend to precipitate. Carmalum made up with 500 c.c. of water instead of 200, and with glycerin or 10 per cent. of formol or pyroligneous acid added, keeps well.

218. Mayer's Aqueous Aluminium-Chloride-Solution (*Mitth. Zool. Stat. Neapel*, x, 1902, p. 490).—Carminic acid, 1 grm.; chloride of aluminium, 3 grms.; water, 200 c.c. Add an antiseptic, as for carmalum.

Use as carmalum. The stain is of a blue-violet colour, very powerful, and elective, but not so purely nuclear as carmalum. It is recommended only as a substitute for carmalum in cases in which the latter is counter-indicated on account of the alum in it or the like.

219. Alum-Carmine and Picric Acid.—Alum-carmine objects may be double-stained with picric acid. LEGAL (*Morph. Jahrb.*, viii, p. 353) combines the two stains by mixing ten vols. of alum carmine with one of saturated picric acid solution. I find this very recommendable.

219a. Aceto-Carmine (Acetic Acid Carmine) SCHNEIDER (*Zool. Anzeig.*, 1880, p. 254).—To boiling acetic acid of 45 per cent. strength add carmine until no more will dissolve, and filter. (Forty-five per cent. acetic acid is, according to Schneider, the strength that dissolves the largest proportion of carmine.)

To use the solution you may either dilute it to 1 per cent. strength, and use the dilute solution for slow staining; or a drop of the concentrated solution mav be added to a fresh preparation under the cover-glass. If you use the concentrated solution it *fixes* and stains at the same time, and hence may render service for the study of fresh objects. It is *very penetrating.* The stain is a pure nuclear one. Unfortunately the preparations cannot be preserved, and for this and other reasons the stain is *of very restricted applicability.*

A similar stain has been prepared with formic acid by PIANESE (see *Zeit. wiss. Mik.*, x, 4, 1894, p. 502).

For BURCHARDT'S pyroligneous-acid carmines see *Arch. mik. Anat.*, liii, 1898, p. 232; and *Jena. Zeit. Naturw.*, xxxiv, 1900, p. 720.

220. Iron Carmine.—I recommend trial of the following, which I have already published in the *Traité des Méth. Techniques,* LEE et HENNEGUY, 1902. Sections (I have not tried material in bulk) are mordanted (a few hours will suffice) in sulphate of iron (Benda's *Liquor ferri,* as for iron hematoxylin), washed, and stained for an hour or so in 0·5 per cent. solution of carminic acid in alcohol of 50 per cent. Wash in alcohol of 50 per cent.; no differentiation is necessary. When successful, an almost pure chromatin stain, quite as sharp as iron hæmatoxylin, but somewhat weak.

221. Iron Carmine.—PFEIFFER VON WELLHEIM (*Zeit. wiss. Mik.*, xv, 1898, p. 123) mordants for six to twelve hours in a very weak solution of chloride of iron in 50 per cent. alcohol, washes in 50 per cent. alcohol, and stains as above. Overstains may be corrected with 0·1 to 0·5 per cent. HCl alcohol. I find this good, but not so good as the last.

222. Iron Carmine (ZACHARIAS, *Zool. Anz.*, 1894, p. 62).—Stain for several hours in an aceto-carmine (made by boiling 1 grm. of carmine with 150 to 200 c.c. of acetic acid of 30 per cent., for twenty minutes, and filtering). Rinse the objects with dilute acetic acid, and bring them (taking care not to touch them with metallic instruments) into a 1 per cent. solution of ammoniated citrate of iron. Leave them, for as much as two or three hours if need be, till thoroughly penetrated and blackened (with sections this happens in a few minutes). Wash for several hours in distilled water. A chromatin and plasma stain.

223. Iron Carmalum (DE GROOT, *Zeit. wiss. Mik.*, xx, 1903, p. 21).—Dissolve 0·1 grm. of ferric alum in 20 c.c. distilled water and add 1 grm. carminic acid. Dissolve, add 180 c.c. of water, warm, add 5 grms. potash alum, dissolve, cool, filter, and add 2 drops of hydrochloric acid. To be used as carmalum, and said to give a stronger strain.

224. Iron Cochineal (SPULER, *Encyclopædie d. mik. Technik*, 1903, p. 153, and 1910, p. 240).—Stain for 48 hours in a stove, in extract of cochineal (made in a highly complicated way), wash with water, put into solution of ferric alum of ¾ per cent. strength for 24 hours or more. If the stain is not sufficiently intense, the whole process may be repeated.

PETER (*Zeit. wiss. Mik.*, xxi, 1904, p. 314) stains material in bulk for 48 hours (sections 18 to 24) in an incubator, in a similar extract, acidified with HCl, treats with iron-alum of 2½ per cent. for one hour to one day (sections half to two minutes), then alcohol, xylol, paraffin, or balsam. Chromatin black, protoplasm grey, *yolk granules red.*

HANSEN (*ibid.*, xxii, 1905, p. 85) stains sections or entire objects in a solution of 5 to 10 grms. cochineal, 8 grms. ferric alum, 250 c.c. water, and 25 c.c. sulphuric acid of 10 per cent., boiled for fifteen to twenty minutes.

β. *So-called "Neutral" and Alkaline.*

225. Ammonia-Carmine.—Best made by the method of RANVIER. Make a simple solution of carmine in water with a *slight* excess of ammonia, and expose it to the air in a deep crystallising dish until it is entirely dried up. It should be allowed to putrefy if possible. Dissolve the dry deposit in pure water, and filter.

VAN WIJHE (*Vers. Akad.*, Amsterdam, viii, Deel, p. 507) takes an old strong solution of carmine in ammonia (or boils carmine with ammonia and peroxide of hydrogen), then precipitates it by adding alcohol to excess, washes the precipitate with alcohol, and dries it.

226. Soda-Carmine appears to be still used by some for central nervous system (see CUCCATI, *Zeit. wiss. Mik.*, iv, 1887, p. 50). It can be obtained from GRÜBLER & HOLLBORN (*Natron-Carmin*).

226a. **Orth's Lithium-Carmine** (see *early editions*) macerates strongly, and is superfluous. For that of Best, see *Zeit. wiss. Mik.*, xxiii, 1906, p. 322.

227. **Magnesia-Carmine** (Mayer, *Zeit. wiss. Mik.*, xiv, 1897, p. 23). —Take 1 grm. carmine, 0·1 grm. magnesia usta, and 50 c.c. distilled water, boil for five minutes, filter, and add three drops of formol. This is the *stock* solution. A *weak* solution may be made by boiling 0·1 grm. carmine for half an hour in 50 c.c. of magnesia water (made by leaving 0·1 grm. of magnesia usta in contact with 100 c.c. of spring water for a week with frequent agitation, and decanting when required for use). Said to be less injurious to tissues than the other alkaline carmines.

228. **As to Picro-carmine.**—The term "picro-carmine" is commonly used to denote a whole tribe of solutions in which carmine, ammonia, and picric acid exist *uncombined* in haphazard proportions. These solutions do *not* contain a double salt of picric and carminic acid and ammonia, or *picro-carminate of ammonia*. They are always alkaline, and frequently injurious to tissues. The *raison d'être* of picro-carmine does not lie in its capacity of affording a double stain, but in that the picric acid in it is supposed to neutralise the ammonia, which it only does imperfectly. See Mayer in *Zeit. wiss. Mik.*, xiv, 1897, p. 18.

229. **Ranvier's Picro-carmine, Original Formula** (*Traité*, p. 100). —To a saturated solution of picric acid add carmine (dissolved in ammonia) to saturation. Evaporate down to one fifth the original volume in a drying-oven, and separate by filtration the precipitate that forms in the liquid when cool. Evaporate the mother liquid to dryness, and you will obtain the picro-carmine in the form of a crystalline powder of the colour of red ochre. It ought to dissolve completely in distilled water; a 1 per cent. solution is best for use.

For slow staining, dilute solutions may advantageously have 1 or 2 per cent. of chloral hydrate added to them.

Overstains may be washed out with hydrochloric acid, say 0·5 per cent. in water, alcohol, or glycerin.

Preparations should be mounted in balsam, or if in glycerin, this should be acidulated with 1 per cent. of acetic acid, or better, formic acid.

Ranvier's Newer Formula does not give a more constant product (see *previous editions*).

230. Van Wijhe dissolves 0·5 per cent. of the dry ammonia-carmine, § 225, in a 1 per cent. solution of neutral picrate of ammonia, boils until the vapour ceases to blue reddened litmus paper, and adds 1 per cent. of chloral hydrate. Gives an almost neutral preparation.

231. **Mayer's Picro-magnesia Carmine** (*Zeit. wiss. Mik.*, xiv, 1897, p. 25) is relatively constant and innocuous to tissues. It consists of 1 vol. of the *stock* solution of magnesia-carmine (§ 227), and 10 vols. of a 0·6 per cent. solution of picrate of magnesia, or of equal parts of the *weak* solution and the picrate solution. The picrate may be obtained from Grübler & Hollborn, or the solution may be made by heating 0·25 grms. of carbonate of magnesia in 200 c.c. of 0·5 per cent. solution of picric acid, allowing to settle, and filtering.

De Groot's picro-magnesia carmine (*ibid.*, xxix, 1912, p. 184) contains ammonia, which is bad, and seems to me superfluous.

232. **Other Formulæ for Picro-carmine and Other Aqueous Carmines (Acid and Alkaline).**—I have tried most of them, and found no real advantage in any of them (see *previous editions*).

B. Alcoholic Carmine Stains.

233. **Alcoholic Borax-carmine** (Grenacher, *Arch. mik. Anat.*, xvi, 1879, p. 466, *et seq.*).—Make a *concentrated* solution of carmine in borax solution (2 to 3 per cent. carmine to 4 per cent. borax) by boiling for half an hour or more (or allowing it to stand, with occasional stirring, for two or three days); dilute it with about an equal volume of 70 per cent. alcohol, allow it to stand some time and filter.

Preparations should remain in the stain until they are thoroughly penetrated (for days if necessary), and then be brought (*without first washing out*) into alcohol *of 70 per cent.* acidulated with 4 to 6 drops of hydrochloric acid to each 100 c.c. of alcohol. They are left in this until they have taken on a bright transparent look (which may require days), and may then be washed or hardened in neutral alcohol. Four drops of HCL is generally enough. Three drops I find not quite sufficient.

For delicate objects, and for very impermeable objects, it may be well to increase the proportion of alcohol in the stain; it may conveniently be raised to about 50 per cent. It should not exceed 60 per cent. in any case (Mayer).

This stain used to be the most popular of any for staining in bulk. It is easy to use, and gives a most splendid coloration. But it is not so penetrating as is commonly supposed, and has the defect of sometimes forming precipitates in the cavities of bulky objects which cannot be removed by washing out. And the fluid is alkaline, and therefore may not be suitable for certain delicate work.

234. Mayer's Paracarmine (*Mitth. Zool. Stat. Neapel*, x, 3, 1892, p. 491).—Carminic acid, 1 grm.; chloride of aluminium, 0·5 grm.; chloride of calcium, 4 grms.; 70 per cent. alcohol, 100 c.c. Dissolve cold or warm, allow to settle, and filter.

Objects to be stained *should not have an alkaline reaction*, nor contain any considerable amount of carbonate of lime (spicules or skeletal parts of corals, etc.) which would give rise to precipitates. Wash out sections or objects intended to be sectioned, with pure 70 per cent. alcohol. Objects intended to be mounted whole may be washed out with a weak solution of aluminium chloride in alcohol, or if this be not sufficient, with 5 per cent. common acetic acid (or 2·5 per cent. glacial acetic acid) in alcohol. This may also be done with section material, if it is desired to obtain a more purely nuclear stain.

For staining bulky objects with large cavities, such as *Salpa*, the solution should be diluted (with alcohol); and as this may cause precipitates to form during the staining, especially if the objects are not very clean, it is advisable to *slightly acidify the dilute solutions*.

Instead of calcium chloride, which is very hygroscopic, strontium chloride may be taken.

Paracarmine is less hurtful to delicate tissues than borax carmine; it is more highly alcoholic, therefore more penetrating; and has less tendency to form precipitates in the interior of objects. But, in my hands, it does not give quite so fine a stain.

234a. **Alcoholic Hydrochloric-Acid Carmine.**—Grenacher's receipt (*Arch. f. Mik. Anat.*, xvi, 1879, p. 468) is troublesome. That of Mayer (*Mitth Zool. Stat. Neapel*, iv, 1883, p. 521; *Intern. Monatsschr. f. Anat., etc.*, 1897, p. 43) is better: Carmine 4 grms.; water, 15 c.c.; hydrochloric acid, 30 drops. Boil till the carmine is dissolved, add 95 c.c. of 85 per cent. alcohol, and neutralise by adding ammonia until the carmine begins to precipitate.

If it be desired to dilute the solution, it should be done with alcohol, not water, and alcohol of 80 to 90 per cent. should be taken for washing out.

A very *powerful* stain, which I have found useful. If it be desired to have a purely nuclear stain, the alcohol must be *very slightly* acidulated with HCl.

For a complicated receipt of Loewenthal see *Zeit. wiss. Mik.*, xix, 1902, p. 56.

235. Alcoholic Cochineal, Mayer's Old Formula (*Mitth. Zool. Stat. Neapel.* ii, 1881, p. 14).—Cochineal in coarse powder is macerated for several days in alcohol of 70 per cent. For each gramme of the cochineal there is required 8 to 10 c.c. of the alcohol. Stir frequently. Filter.

The objects to be stained must previously be saturated with alcohol of 70 per cent., and alcohol of the same strength must be used for washing out or for diluting the staining solution. The washing out must be repeated with fresh alcohol until the latter takes up no more colour. Warm alcohol acts more rapidly than cold. Overstaining seldom happens; it may be corrected by means of 70 per cent. alcohol, containing $\frac{1}{10}$ per cent. hydrochloric or 1 per cent. acetic acid.

Small objects and thin sections may be stained in a few minutes; larger animals require hours or days.

A nuclear stain, slightly affecting protoplasm. The colour varies with the reaction of the tissues, and the presence or absence of *certain salts* in them. Crustacea with thick chitinous integuments are generally stained red, most other organisms blue. The stain is also often of different colours in different tissue elements of the same preparation. Glands or their secretion often stain grey-green.

Acids lighten the stain and make it yellowish-red. Caustic alkalies turn it to a deep purple.

All acids must be carefully washed out from the objects before staining, or a diffuse stain will result. The stain is permanent in oil of cloves and balsam.

Very penetrating and especially useful for Arthropoda.

It has over the new fluid (next §) the advantage of being more highly alcoholic; and it does not contain free acid, so that it *can be used with calcareous structures* which it is wished to preserve—which the new fluid cannot. For specimens of *Pluteus*, for instance, I find it excellent. But it only gives *good* results with such objects as contain the necessary salts, § 212.

236. Mayer's Alcoholic Cochineal, New Formula (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 498).—Cochineal, 5 grms.; chloride of calcium, 5 grms.; chloride of aluminium, 0·5 grm.; nitric acid of 1·20 sp. gr., 8 drops; 50 per cent. alcohol, 100 c.c. Powder the cochineal and rub up with the salts, add the alcohol and acid, heat to boiling-point, leave to cool, leave for some days standing with frequent agitation, filter.

Use as the old tincture, the objects being prepared and washed out with 50 per cent. alcohol. Mayer only recommends it as a *succedaneum* of paracarmine.

Since this fluid contains in itself all the necessary salts (§ 212), it gives good results with *all classes of objects.*

CHAPTER XIII.

HÆMATEIN (HÆMATOXYLIN) STAINS.

237. **Introduction.**—*Hæmatoxylin* is a dye extracted from logwood. It is a substance that oxidises very readily, thus becoming converted into *hæmatein,* or, as often happens, into other more highly oxidised products. It appears to be now thoroughly well established (see Nietzki, *Chemie der organischen Färbstoffe,* Berlin, Springer, 1889, pp. 215—217, and Mayer, *Mitth. Zool. Stat. Neapel,* x, 1891, p. 170) that the colouring agent in solutions of logwood or hæmatoxylin is not the hæmatoxylin itself, but hæmatein formed in them (or, in some cases, one of the higher oxidation products).

Hæmatein is an acid body, a " colour acid " (§§ 203, 205). Substantively employed, it is a very weak plasma stain. But combined with appropriate mordants it becomes basophilous, and can be made to give a powerful nuclear stain, or at the same time a nuclear and a selective plasma stain. The mordants employed in histology are aluminium, chrome, iron, copper, and (rarely) vanadium and molybdenum. Aluminium and iron are the mordants most employed, the former furnishing lakes used for progressive staining of material in bulk, the latter forming in most cases in the tissues a lake that requires differentiation, and is only applicable to the staining of sections.

The presence of a sufficient amount of hæmatein in staining solutions was formerly brought about by allowing solutions of hæmatoxylin to oxidate spontaneously by exposure to air. The change thus brought about in the solutions is known as " ripening," and until it has taken place the solutions are not fit to use for staining.

It was discovered by Mayer and Unna independently (see Mayer in *Mitth. Zool. Stat. Neapel,* x, 1891, pp. 170—186; Unna in *Zeit. wiss. Mik.,* viii, 1892, p. 483) that nothing is easier than to bring about this change artificially; all that is necessary being, for instance, to add to a solution of hæmatoxylin containing alum a little neutralised solution of peroxide of hydrogen or other powerful oxidising agent.* The solution becomes almost instantaneously dark blue, "ripe" and fit for staining. Other methods of "ripening," or of preparing hæmatein separately, are given further on, and constitute a great progress. For under the old practice of leaving staining solutions to "ripen" by the action of the air, it is necessary to wait for a long time before the reaction is obtained. During all this time, it may be weeks or months, there is no means, except repeated trial, of ascertaining whether the solution at any moment contains sufficient hæmatein to afford a good stain. And here a second difficulty arises: the oxidising process continuing, the solutions become "over-ripe"; the hæmatein, through further oxidation, passes over into colourless compounds, and the solutions begin to precipitate. They are therefore, in reality, a mixture in constantly varying proportions of "unripe," "ripe," and "overripe" constituents (the first and last being useless for staining purposes), and, in consequence, their staining power is very inconstant.

Logically, therefore, as concluded by Mayer, not hæmatoxylin, but *hæmatein,* should be taken in the first instance for making the staining solution. This at once relieves us from the tedious and uncertain process of "ripening" in the old way. We have thus a ripe solution to begin with.

But this is not always indicated; for such solutions may easily over-oxidise, either in the bottle or on contact with the tissues. So that it is sometimes preferable to start from hæmatoxylin. In this case, it should not be done by dissolving the hæmatoxylin straight away in the other ingredients of the staining solution. The solutions should be made up from a strong *stock solution* made by dissolving hæmatoxylin crystals in absolute alcohol: one in ten is a good proportion. This solution should be kept for a long time—months, at least, a year if possible; it gradually becomes of a vinous red, and should not be used till it has become quite dark. It has then become to a great extent oxidised into hæmatein, and the staining solutions made up from it will be at once fairly ripe.

---

* Re-invented lately (*Zeit. wiss. Mik.*, xxix, 1912, p. 69) by Piazza, who adds to Boehmer's solution about 20 per cent., to Delafield's about 7 per cent., to Ehrlich's about 12 per cent. of peroxide of hydrogen.

Hæmatein (or hæmatoxylin) affords a stronger stain than carmine, and gives better results with tissues fixed in osmic or chromic mixtures. The alum solutions are indicated for staining in bulk, iron hæmatoxylin for sections.

238. **Hæmatoxylin** is found in commerce in the form of crystals, either colourless or browned by oxidation, easily soluble in either water, glycerin, or alcohol.

Hæmatein is found in commerce as a brown powder, entirely, though with difficulty, soluble in distilled water and in alcohol, giving a yellowish-brown solution, which remains clear on addition of acetic acid. Alkalies dissolve it with a blue-violet tint.

MAYER (*Zeit. wiss. Mik.*, xx, 1903, p. 409) prepares it as follows: 1 grm. of hæmatoxylin is dissolved by boiling in not more than 10 c.c. of distilled water, and to the solution is added a hot solution of 0·2 grm. of iodate of sodium in about 2 c.c. of water. Mix well and cool by placing the recipient in cold water. After a couple of hours bring the mixture on to a filter, wash thereon with cold water to remove the excess of iodide of sodium that has formed, then dry the residue.

There is also found in commerce an ammonia-compound of hæmatein—*hæmatein-Ammoniak,* also known as *Hæmateinum crystallisatum*; this may be obtained in a sufficiently pure state from GRÜBLER & HOLLBORN.

This is somewhat more easily soluble in both water and alcohol than hæmatein is, and does quite as well for staining purposes. It can be made as follows:

239. **Hæmateate of Ammonia** (MAYER, *Mitth. Zool. Stat. Neapel,* x, 1891, p. 172).—Dissolve 1 grm. of hæmatoxylin with the aid of heat in 20 c.c. of distilled water, filter if necessary, add 1 c.c. of caustic ammonia (of 0·875 sp. gr.), and bring the liquid into a capsule of such dimensions that its bottom be covered to a depth of not more than half a centimetre. Let the liquid evaporate at the ordinary temperature and be protected from dust. The dry product will consist of hæmateate of ammonia, about equal in weight to the hæmatoxylin taken in the first instance. The evaporation should not be hastened by heat, as this may give rise to the formation of substances that are insoluble in alcohol. The preparation should not be touched, until it is dry, with any other instruments than such as are made of glass, porcelain, or

platinum. The product is not of perfectly constant quality. It ought to dissolve easily in water or alcohol, and the solution should not become turbid on addition of acetic acid; if it does, it is over-oxidised.

240. Iron Hæmatoxylin, Generalities.—This method is due to BENDA (*Verh. Phys. Ges.*, 1885—1886, Nos. 12, 13, 14; *Arch. Anat. Phys.*, 1886, p. 562; *third ed.* of this work, p. 365).

The method was independently worked out about the same time by M. HEIDENHAIN. The method is almost universally practised in the form given by Heidenhain, not on account of any essential difference between the two, for there is none, but chiefly because Heidenhain has given more precise instructions concerning the process.

After carefully comparing Heidenhain's process with Benda's later process (next §), I find that the two give an absolutely identical stain; that is to say, that if you mordant in Benda's *liquor ferri*, next §, and differentiate in the same, you will get exactly the same effect as by mordanting in ferric alum and differentiating in the same. But you may vary the results somewhat by varying the differentiation. Benda has pointed out (*Verh. Anat. Ges.*, xv, 1901, p. 156) that you may differentiate either by an agent which simply dissolves the lake—such as acetic or hydrochloric acid; or by an oxidising agent, such as chromic acid, or the *liquor ferri* or the ferric alum. The former, he thinks, are the best for the demonstration of nuclear structures, the latter for cytoplasmic structures. For these he greatly recommends WEIGERT's borax-ferricyanide mixture, as being the easiest and safest to employ.

For myself, I find that differentiation in the iron salt (§ 241 or § 242) is sufficient for almost all purposes. Acetic acid of 30 per cent. acts much too quickly to be safe, and causes swelling of the tissues.

Van GIESON's picro-säurefuchsin has been recommended as a differentiation fluid by Benda (*Deutsch. med. Wochenschr.*, 1898, No. 30). I find it gives very delicate differentiations, but acts very slowly, requiring nearly as many hours as the iron alum solution does minutes. The addition of the säurefuchsin to the picric acid is, I find, not necessary, and may prove an injurious complication.

In these processes hæmatoxylin is generally used for the stain, *not hæmatein*, the iron salt oxidising it into hæmatein, or into a higher oxidation product. I have obtained some good stains with hæmatein, but also some very bad ones; presumably the solutions easily over-oxidise on contact with the iron salt.

The hæmatoxylin is generally dissolved in water. I frequently prefer alcohol, of 50 per cent., as less injurious to tissues.

The method is a regressive one. It has been proposed to stain progressively, which I have tried, and had extremely bad results.

The differentiation requires to be carefully timed. For this reason the method is only applicable to *sections*, which should be thin, best not over 10 μ.

Iron hæmatoxylin is one of the most important of stains. It enables us to stain elements which cannot be selectively stained in any other way. The stain is very powerful, and of a certain *optical quality* that is peculiarly suited to the employment of high powers; it will allow of the use of deeper eye-pieces than other stains. It will take effect on any material, and is quite permanent. Further details as to the characters of the stain are given in § 242.

241. **Benda's later Iron Hæmatoxylin** (*Verb. d. Anat. Ges.*, vii, 1, 1893, p. 161).—Sections are mordanted for twenty-four hours in *liquor ferri suphurici oxidati*, P.G.,* diluted with one or two volumes of water. They are then well washed, first with distilled water, then with tap water, and are brought into a 1 per cent. solution of hæmatoxylin in water, in which they remain till they have become thoroughly black. They are then washed and differentiated. The differentiation may be done either in 30 per cent. acetic acid, in which case the progress of the decoloration must be watched; or in a weaker acid, which will not require watching; or in the sulphate solution strongly diluted with water.

I find that if the iron solution be taken for the differentiation, it should be taken *extremely* diluted (of a *very pale* straw-colour, about 1 : 30 of water), and the progress of the differentiation watched; as if it be only diluted about tenfold, for instance, the decoloration is extremely rapid. See also last §.

---

* This preparation consists of sulphate of iron, 80 parts; water, 40; sulphuric acid, 15; and nitric acid, 18, and contains 10 per cent. of Fe. Doubtless the *ferri persulphatis liquor* B. P. will do instead; the point is, to have a per-salt, and not a proto-salt.

I also find that Benda's mordant is unnecessarily, sometimes harmfully, strong, and that the *Liquor ferri* may be diluted *tenfold* with advantage. The duration of the bath in the mordant is also for most purposes excessive as directed by Benda. I find that three to six hours in the solution diluted tenfold is generally sufficient, with favourable material.

242. HEIDENHAIN'S **Iron Hæmatoxylin** (M. HEIDENHAIN, "Über Kern und Protoplasma," in *Festschr. für Kölliker*, 1892, p. 118).—Sections are treated from half an hour to at most two or three hours with a 1·5 to 4 per cent. solution of ferric alum (ammonio-ferric sulphate). By this is always meant in histology the double salt of *ammonium* and sesquioxide of iron $(NH_4)_2Fe_2(SO_4)_4$, in clear violet crystals; the double salt of the protoxide, or salt of MOHR in green crystals, will not serve. If the crystals have become yellow and opaque, they have gone bad, and should be rejected. They ought to be kept in a stoppered bottle, and the solution should be made in the cold (*Arch. mik. Anat.*, xliii, 1894, pp. 431, 435). The sections are then washed with water and stained for half an hour in an aqueous solution (of about 0·5 per cent.) of hæmatoxylin. They are then rinsed with water, and again treated with the iron solution, which slowly washes out the stain. The progress of the differentiation ought to be controlled under the microscope. The sections should to this end be removed from time to time from the alum solution, and put into tap-water whilst they are being examined. This is favourable to the stain. As soon as a satisfactory differentiation has been obtained, the preparations are washed for at least a quarter of an hour in running water, but not more than an hour, and mounted. The results differ according to the duration of the treatment with the iron and the stain. If the baths have been of short duration, viz. not more than half an hour in the iron and as much in the stain, *blue* preparations will be

obtained. These show a very intense and highly differentiated stain of nuclear structures, cytoplasmic structures being pale. If the baths in the iron and in the stain have been prolonged (twelve to eighteen hours), and the subsequent differentiation in the second iron bath also duly prolonged, *black* preparations will result. These show chromosomes stained, central corpuscles stained intensely black, cytoplasm sometimes colourless, sometimes grey, in which case achromatic spindle-fibres and cell-plates are stained, connective-tissue fibres black, red blood-corpuscles black, micro-organisms sharply stained, striated muscle very finely shown.

Later (*Zeit. wiss. Mik.*, xiii, 1896, p. 186) Heidenhain gives further instructions for the employment of this stain in the study of central corpuscles. All alcohol should be removed from the tissues,* by means of distilled water before bringing them into the mordant. This should be a 2½ per cent. solution of ferric alum, *not weaker*. Leave the sections therein (fixed to slides by the water method, § 186) for six to twelve hours, or at least not less than three. Keep the slides upright in the mordant, not lying flat. Wash out *well* with water before staining. Stain in a "ripened" hæmatoxylin solution, *i. e.* one that has stood for four weeks [of course, if you make it up with the ripened brown alcoholic solution recommended § 237 *sub. fin.*, this will be superfluous]. Stain from twenty-four to thirty-six hours. *Use the same staining solution over and over again* until it becomes spoilt; for the solution after having been used gives a more energetic stain, owing to its containing a trace of iron brought over by the sections. Differentiate in a 2½ per cent. solution of ferric alum. Rinse for ten minutes in running water, clear with xylol, *not* with any essential oil, and mount in xylol-balsam. See also under "Centrosomes."

BIELASZEWICS (*Bull. Acad. Cracovie*, 1909, 2 série, p. 152) differentiates with very weak solution of calcium chloride; GUARNIERI (*Mon. Zool. Ital.*, xvii, 1906, p. 44) with saturated solution of picric acid.

GURWITSCH (*Zeit. wiss. Mik.*, xviii, 1902, p. 291) floods sections on the slide with mordant, warms on a water-bath till bubbles are given off or the mordant becomes turbid, then stains with the hæmatoxylin in the same way. The whole process takes about ten minutes.

---

* Why? I find my iron-alum solution, as well as the *liquor ferri sulph. oxid.*, last §, mix clear with alcohol without the least precipitate forming.

Held (*Arch. Anat. Phys., Anat. Abth.*, 1897, p. 277) adds to the staining bath a very little of the iron-alum solution until a scarcely perceptible precipitate is produced. A dangerous practice. I find it is not even safe to add a little of an over-used bath (*supra*).

Francotte (*Arch. Zool. Expér.*, vi, 1898, p. 200) mordants with *tartrate* of iron, Mallory (*Journ. Exper. Med.*, v, 1900, p. 15) with chloride.

243. Iron Hæmatoxylin (Bütschli, *Unters. über mikroskopische Schäume u. das Protoplasma*, etc., 1892, p. 80).—Sections treated with a weak brown aqueous solution of ferric acetate, washed with water, and stained in 0·5 per cent. aqueous solution of hæmatoxylin. A stain of extraordinary intensity, used by Bütschli for sections, 1 μ in thickness, of Protozoa.

244. Weigert's Iron Hæmatoxylin Mixture (*Zeit. wiss. Mik.*, xxi, 1904, p. 1).—Mix one part of a 1 per cent. solution of hæmatoxylin in alcohol of 96 per cent. with one of a solution containing 4 c.c. of *liq. ferri sesquichlor.*, 1 c.c. of officinal hydrochloric acid (sp. gr. 1·124) and 95 of water. The mixture may be kept for some days (until it begins to smell of ether), but is *best used fresh*. Stain sections for a few minutes; no differentiation is necessary.

For an earlier process of Weigert's (*Allg. Zeit. Psychiatr.*, 1894, p. 245) see *last edition*.

Morel and Bassal (*Journ. Anat. Phys.*, xlv, 1909, p. 632) stain *in bulk* in Weigert's mixture with the addition of 1 c.c. of 4 per cent. solution of acetate of copper.

245. Janssens' Iron Hæmatoxylin (**"Hématoxyline noire"**; *La Cellule*, xiv, 1897, p. 207).—A similar mixture to that of Delafield, ferric alum being taken instead of ammonia alum, the rest as in Delafield's. A progressive stain, nuclear: for yeast cells.

246. Hansen's Iron Hæmatoxylin (*Zeit. wiss. Mik.*, xxii, 1905, p. 55).—A solution of 10 grms. ferric alum in 150 c.c. water is added to a solution of 1·6 grm. hæmatoxylin in 75 c.c. water, the mixture heated to boiling-point and cooled without access of air. Filter before use. To get a pure nuclear stain, add dilute sulphuric acid.

247. Aluminium Hæmatein (Alum Hæmatoxylin) Generalities.—The mordant and dye are generally combined in a single staining bath, giving a *progressive* stain. The stain is in different tones of blue or red according to the composition of the staining solution. Neutral or alkaline solutions give a blue stain; acid solutions give a red one. In order to *get a blue stain* in preparations that have come out red through the acidity of the staining bath, it is a common practice to treat them with weak ammonia, in the belief that the blue colour is restored by neutralisation of the acid that is the cause of the redness. According to Mayer, the ammonia acts, not by neutralising the acid, but by precipitating the alumina, which carries down the hæmatein with it (if no alumina were present the colour would be purple, not blue).* The *same result* can generally be obtained by merely washing out with common tap-water, which is usually sufficiently alkaline, and can be obtained with certainty by treatment with bicarbonate of soda or acetate of soda or potash. And this is the preferable course, as ammonia is certainly a dangerous thing to treat delicate tissues with. Of course this is a different question from that of *neutralising* with an alkali tissues that have been treated with an acid to correct over-staining. Here the neutralisation may be indicated in the interest of the *preservation* of the stain.

Squire (*Methods*, p. 22) finds that sections can be blued in a few seconds by treatment with a 1 : 1000 solution of bicarbonate of soda in distilled water. Mayer holds that acetate of potash is the most inoffensive reagent to take ; a strength of 0·5 to 1 per cent. may be taken.

Several of these solutions have a great tendency to over-stain. Over-stains may be corrected by washing out with weak acids (*e. g.* 0·1 to 0·2 or even 0·5 per cent. of hydrochloric acid, or with oxalic or tartaric acid), but this is not favourable to the permanence of the stain. Carnoy (*La Cellule*, xii, 2, 1897, p. 215) recommends iodised water. If acids be used, it is well to neutralise afterwards with ammonia or bicarbonate of soda (0·1 per cent.).

Bicarbonate of soda may be used for neutralisation with 70 per cent. *alcohol* as the vehicle (von Wistinghausen, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 41).

Over-staining may be avoided by staining very slowly in dilute solutions. The purest chromatin stains are obtained by staining for a short time (sublimate sections half an hour, say) in solutions of *medium* strength, such as hæmalum diluted ten to twenty-fold with water. The stain obtained either with very strong solutions, or with the slow stain of the dilute solutions, is at the same time a plasma-stain, which of course may or may not be desired. MAYER says that very dilute solutions will give a pure nuclear stain if they have been diluted with *alum-solution*, or have been *acidified*. Chromosmium material will not yield a pure chromatin stain unless it is very *fresh*; it is consequently next to impossible to obtain the reaction with paraffin sections of such material; they constantly give a plasma-stain in addition to the chromatin stain, which is not the case with sublimate material.

---

* Fischer, in his *Fixirung, Färbung u. Bau des Protoplasmus*, pp. 156, 157, does not admit this explanation. He proposes another one of a highly speculative nature.

The stain is fairly permanent in balsam, but is very liable to fade a little, and may fade a great deal. If acids have been used after staining, great care should be taken to wash them out thoroughly before mounting. In aqueous media the stain cannot be relied on to keep (this refers to the old solutions: MAYER finds that his hæmatein preparations have kept well for at least some months in glycerin, if not acid, and, with certain precautions, in balsam). Turpentine-balsam should not be used.

Formulæ §§ 248 to 259 give *aqueous* solutions; and §§ 260 to 263 *alcoholic* ones.

248. MAYER'S **Hæmalum, Newer Formula** (*Zeit. wiss. Mik.*, xx, 1903, p. 409).—*Hæmatoxylin*, 1 grm.; water, 1 litre. Dissolve, and add 0·2 grm. of iodate of sodium ($NaIO_3$) and 50 grms. of alum, dissolve and filter.

This is an amended formula. The original one (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 172) was: One grm. of *hæmatein* (or the ammonia salt, §§ 238, 239) dissolved with heat in 50 c.c. of 90 per cent. alcohol, and added to a solution of 50 gr. of alum in a litre of distilled water.

This solution does not keep very well, but may be made more stable by adding 50 grms. of chloral hydrate and 1 grm. of citric (or acetic) acid.

It stains equally well, *either at first, or later*. Concentrated, it stains sometimes almost instantaneously, or in any case very rapidly. (Spring water or tap-water containing lime must not be used for diluting; perhaps weak solution of alum in distilled water is the best means of all.) After staining, sections may be washed out either with distilled or common water. It is *admirable for staining in bulk.* Large objects will, however, require twenty-four hours' staining, and should be washed out for the same time (this should be done with 1 per cent. alum solution if a sharp nuclear stain be desired). All alum must be carefully washed out of the tissues before mounting in balsam; and it is well to blue the stain with tap-water or otherwise, § 257. The stain is generally a nuclear one; in any case such may be obtained by washing out with alum-solution. Mayer's preparations have kept well in glycerin (care being taken not to have it acid), also in balsam. If oil of bergamot be used for clearing, it must be thoroughly removed by means of oil of turpentine before mounting, and oil of cloves is dangerous. It is best (Mayer, *in litt.*) to use only xylol, benzol, or chloroform, and to mount in xylol-balsam or chloroform-balsam or benzol-balsam.

Hæmalum may be mixed with alum-carmine, Säurefuchsin, or the like, to make a double staining mixture; but it seems preferable to use the solutions in succession.

249. Mayer's **Acid Hæmalum** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 174).—This is hæmalum with 2 per cent. glacial acetic acid (or 4 per cent. common acetic acid). To be used as the last, washing out with ordinary water in order to obtain a blue-violet tint of stain. The solution keeps better.

250. Unna's **Half-ripe Constant Stock Solution** (*Zeit. wiss. Mik.*, viii, 1892, p. 483).

Hæmatoxylin	1
Alum	10
Alcohol	100
Water	200
Sublimed sulphur	2

If the sulphur be added to the hæmatoxylin solution only when the latter has become somewhat strongly blue, *i. e.* after two or three days' time, the stage of oxidation attained by the solution will be fixed for some time by the sulphur, and according to Unna the solution will remain "constant" in staining power. Mayer (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 309) finds that the sulphur process does not preserve the solutions for long, whilst *glycerin* does; see below, "Glychæmalum."

251. Mayer's **Glychæmalum** (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 310).—Hæmatein (or hæmateate of ammonia) 0·4 grm. (to be rubbed up in a few drops of glycerin); alum, 5 grms.; glycerin, 30; distilled water, 70. The stain is *not purely nuclear*, but may be made so by

washing out with alum solution or a weak acid. The solution *keeps admirably*.

RAWITZ (*Leitfaden*, 2nd ed., p. 63) takes 1 grm. hæmatein, 6 grms. ammonia alum, 200 grms. each of water and glycerin.

Or (*Zeit. wiss. Mik.*, xxv, 1909, p. 391) 1 grm. hæmatein, 10 grms. of nitrate of aluminium, 250 grms. each of water and glycerin.

252. HANSEN'S Solution (*Zool. Anz.*, 1895, p. 158).—See *fourth edition*. Hansen oxidises a mixture of alum and hæmatoxylin by means of permanganate of potash. I find it does not keep. See also MAYER in *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 309, or the *Grundzüge*, LEE and MAYER, 1901, p. 171.

253. HARRIS'S Solution (*Micr. Bull.*, xv, 1898, p. 47; *Journ. App. Mic.*, iii, p. 777).—Alum-hæmatoxylin solution ripened by addition of mercuric oxide. MAYER (*Grundzüge*, 1901, p. 171) finds the formula "gives too much hæmatein."

254. Böhmer's Hæmatoxylin (*Arch. mik. Anat.*, iv, 1868, p. 345; *Aerzt. Intelligenzbl., Baiern.*, 1865, p. 382).—Make (A) a solution of hæmatox. cryst. 1 part, alcohol (absolute) 12 parts and (B) alum 1 part, water 240. For staining, add two or three drops of A to a watch-glassful of B.

The alcoholic solution of hæmatoxylin ought to be *old and dark* (§ 237).

255. Delafield's Hæmatoxylin (*Zeit. wiss. Mik.*, ii, 1885, p. 288; frequently attributed erroneously to GRENACHER or PRUDDEN).—To 400 c.c. of saturated solution of ammonia-alum (that is about 1 to 11 of water) add 4 grms. of hæmatox. cryst. dissolved in 25 c.c. of strong alcohol. Leave it exposed to the light and air in an unstoppered bottle for three or four days. Filter, and add 100 c.c. of glycerin and 100 c.c. of methylic alcohol (CH_4O). Allow the solution to stand (uncorked) until the colour is sufficiently dark, then filter.

This solution keeps for years. It is well to allow it to ripen for at least two months before using it.

For staining, enough of the solution should be added to pure water to make a very dilute stain. It is an extremely powerful stain.

It is still much used. I find that when *well ripened*—for years rather than months—it is quite a first-class stain.

BÜTSCHLI (*Unters. üb. mikroscopische Schäume u. das Protoplasma*, etc., 1892) recommends, under the name of "acid hæmatoxylin," solution of Delafield very strongly diluted, and with enough acetic acid added to it to give it a decidedly red tint. This gives a sharper and more differentiated nuclear stain than the usual solution.

Martinotti (*Zeit. wiss. Mik.*, xxvii, 1910, p. 31) makes it up with 0·2 per cent. of *hæmatein*, and less alum (2 per cent.).

256. **Ehrlich's Acid Hæmatoxylin** (*Zeit. wiss. Mik.*, 1886, p. 150).—Water 100 c.c., absolute alcohol 100, glycerin 100, glacial acetic acid 10, hæmatoxylin 2 grms., alum in excess.

Let the mixture ripen in the light (with occasional admission of air) until it acquires a dark red colour. It will then *keep*, with constant staining power, *for years*, if kept in a well-stoppered bottle. It is very appropriate for staining in bulk, as over-staining does not occur. I find it excellent.

Mann (*ibid.*, xi, 1895, p. 487) makes up this stain with an equal quantity of hæmatein instead of hæmatoxylin.

Mayer (*Grundzüge*, Lee and Mayer, first edition, p. 154) finds that this is too much and makes the mixture overstain; 0·4 grm. of hæmatein is quite enough.

257. Burchardt's **Pyroligneous Acid Hæmatoxylin** (*Arch. mik. Anat.*, liii, 1898, p. 232) would seem to be superfluous at least.

258. Unna's **Oxidised Hæmatoxylin** (from Martinotti, *Zeit. wiss. Mik.*, xxvii, 1910, p. 31).—Hæmatoxylin 0·5, alum 2, water 60, alcohol 10, glycerin 20, peroxide of hydrogen solution 10, carbonate of soda 0·05.

Martinotti, *loc. cit.*, makes it up with *hæmatein* (0·2 grms).

259. Apáthy's **Hæmatein Mixture I A** (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 712).—Make (a) a solution of 9 per cent. alum, 3 per cent. glacial acetic acid, and 0·1 per cent. salicylic acid in water, and (b) a 1 per cent. solution of hæmatoxylin in 70 per cent. alcohol, preserved for six to eight weeks in a bottle not quite full. Mix one part of a with one of b and one of glycerin. Stains either sections or material in bulk. Apáthy uses it for staining neuro-fibrils.

260. Kleinenberg's **Hæmatoxylin** (*Quart. Journ. Micr. Sci.*, lxxiv, 1879, p 208).—Highly irrational and very inconstant in its composition

and its effects; see early editions; also the criticism of MAYER (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 174), and that of SQUIRE in his *Methods and Formulæ*, p. 25, and the alternative formulæ of SQUIRE (*loc. cit.*) and of VON WISTINGHAUSEN (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 41).

261. MAYER'S **Hæmacalcium** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 182).—Hæmatein (or hæmateate of ammonia, §§ 238, 239), 1 grm.; chloride of aluminium, 1 grm.; chloride of calcium, 50 grms.; glacial acetic acid, 10 c.c. (or common acetic acid, 20 c.c.); 70 per cent. alcohol, 600 c.c. Rub up finely together the first two ingredients, add the acid and alcohol, dissolve either cold or with heat; lastly add the chloride of calcium.

If the objects stain in too red a tone they may be treated with a solution (of about 2 per cent.) of chloride of aluminium in 70 per cent. alcohol, or with a 0·5 to 1 per cent. solution of acetate of soda or potash in absolute alcohol; but washing with neutral alcohol will generally suffice.

With certain objects this solution does not penetrate well. This may be remedied by acidifying the solution, or, which is better, by leaving the objects for some time before staining in acid alcohol. Anyway objects ought NOT to have an alkaline reaction. If these precautions be taken, it will not be necessary to use acid for washing out.

The solution is not recommended as giving as good results as hæmalum, and Mayer recommends it merely as a substitute for Kleinenberg's, in cases in which an *alcoholic* hæmatein stain seems indicated, as being easy to prepare, and constant in its effects.

262. MAYER'S **Hæmastrontium** (*Grundzüge*, LEE and MAYER, 1910, p. 166).—1 grm. hæmatein, 1 grm. aluminium chloride, 50 grm. strontium chloride, 600 c.c. alcohol of 70 per cent., and (if desired) 0·25 g. citric acid. Prepare and use as hæmacalcium.

263. DE GROOT'S **Alcoholic Hæmalum** (*Zeit. wiss. Mik.*, xxix, 1912, p. 182).—Mix 20 c.c. of glycerin with 240 of alcohol of 70 per cent. Take 4 c.c. of the mixture, 2 c.c. of hydrogen peroxide, and 0·5 grm. of hæmatoxylin, and dissolve with heat. Add 60 c.c. of the mixture, 4 grms. of calcium chloride, and 2 grms. of sodium bromide. Dissolve, add 3 grms.

of alum, heat and add 100 c.c. of the mixture. When the alum is dissolved add 0·2 grm. of ferri-cyanide of potassium; dissolve and add 3 grms. more of alum and the rest of the mixture. Said to stain almost as well as hæmalum. Wash out with alcohol of 70 per cent.

284. **Other Alumina-Hæmatein Solutions.**—A large number of suppressed receipts will be found given in the *earlier editions.*

265. R. HEIDENHAIN'S **Chrome Hæmatoxylin** (*Arch. mik. Anat.*, xxiv, 1884, p. 468, and xxvii, 1886, p. 383).—Stain for twelve to twenty-four hours in a $\frac{1}{3}$ per cent. solution of hæmatoxylin in distilled water. Soak for the same time in a 0·5 per cent. solution of neutral chromate of potash. Wash out the excess of chromate with water.

Objects that have been fixed in corrosive sublimate ought to be very carefully washed out with iodine, or the like, as neutral hæmatoxylin forms a black precipitate with any excess of sublimate that may remain in the tissues (see TORNIER, in *Arch. mik. Anat.*, 1886, p. 181).

The process is *adapted to staining in bulk.* You can decolour the objects to any extent by prolonging the soaking in the chromate. Bichromate will do instead of the neutral chromate.

266. APÁTHY'S **Modification of Heidenhain's Process** (*Zeit. wiss. Mik.*, v, 1888, p. 47).—This is an *alcoholic* method. Stain in a 1 per cent. solution of hæmatoxylin in 70 or 80 per cent. alcohol. Differentiate sections of 10 to 15 μ, half the time of staining, sections of 25 to 40 μ twice the time of staining, in 1 per cent. solution of bichromate of potash in 70 to 80 per cent. alcohol, and wash out in alcohol of 70 per cent. All these processes should be done *in the dark.*

For celloidin series of sections, Apáthy (*ibid*, 1889, p. 170) stains in the hæmatoxylin solution as above for ten minutes; then removes the excess of hæmatoxylin fluid from the sections by means of blotting-paper, and brings the series for five to ten minutes into 70 per cent. alcohol containing only a few drops of a strong (5 per cent.) solution of bichromate.

267. SCHULTZE'S **Chrome Hæmatoxylin** (*Zeit. wiss. Mik.*, xxi, 1904, p. 5).—The tissues to be fixed for twelve or more hours in a bichromate or chromic acid solution, preferably an osmium-bichromate mixture or liquid of Flemming. Then to be washed out for twenty-four hours in 50 per cent. alcohol in the dark and stained for twenty-four hours or more in 0·5 per cent. hæmatoxylin in alcohol of 70 per cent., then washed out in alcohol of 80 per cent.

268. HANSEN'S **Chrome Hæmatoxylin** (*ibid.*, xxii, 1905, p. 64).—Ten grms. of chrome alum boiled in 250 c.c. of water till green, and 1 grm. hæmatoxylin (dissolved in 15 c.c. of water) added; to the mixture when cold add 5 c.c. of sulphuric acid of 10 per cent. and (drop by drop) a solution of 0·55 grm. of bichromate of potash in 20 c.c. of water. Filter before use. Wash out with water free from air.

269. **Vanadium Hæmatoxylin** (HEIDENHAIN, *Encyclop. mik. Technik.*, 1903, p. 518).—Add 60 c.c. of a 6 per cent. solution of hæmatoxylin to a 0·25 per cent. solution of vanadate of ammonium (quantity not stated; should be 30 c.c., see COHN in *Anat. Hefte*, xv, 1895, p. 302). The mixture to be used after three or four days; it will not keep over eight days. To be used with sections of sublimate material. A strong *plasma* stain for special purposes, especially mucus glands.

270. BENDA'S **Copper Hæmatoxylin** (*Arch. mik. Anat.*, xxx, 1887, p. 49).—See fourth edition. According to my experience, not to be compared with iron hæmatoxylin, and superfluous.

271. MALLORY'S **Phospho-molybdic Acid Hæmatoxylin** (*Anat. Anz.*, 1891, p. 375).—One part 10 per cent. phospho-molybdic acid solution, 1 part hæmatoxylin, 100 parts water, and 6 to 10 parts chloral hydrate. Let the solution ripen for a week in sunlight, and filter. *Chiefly* for central nervous system. Sections should be stained for from ten minutes to one hour, and washed out in two or three changes of 40 to 50 per cent. alcohol. It is necessary that the solution should be *saturated* with hæmatoxylin in order to obtain the best results; if a good stain be not obtained at once, more hæmatoxylin must be added. Water must never be used for diluting it.

See also RIBBERT (*Centralb. allg. Path.*, vii, 1896, p. 427; *Zeit. wiss. Mik.*, xv, 1898, p. 93), PATELLANI (*Mon. Zool. Ital.*, xiii, 1902, p. 6), and GOLOVIN (*Zeit. wiss. Mik.*, xix, 1902, p. 184).

SARGENT (*Anat. Anz.*, xv, 1898, p. 214) quotes this stain, preceded by mordanting for twenty-four hours in 5 per cent. sulphate of copper, as KENYON'S.

KODIS (*Arch. mik. Anat.*, lix, 1901, p. 211) takes hæmatoxylin, 1 part; molybdic anhydride, 1·5; water, 100; H_2O_2, 0·5, or a crystal of HgO.

POLICE (*Arch. Zool. Napoli*, iv, 1909, p. 300) takes 0·35 grm. hæmatoxylin, 10 drops phospho-molybdic acid of 10 per cent., 10 grms. chloral hydrate, and 100 grms. alcohol of 70 per cent.

272. MALLORY'S **Phospho-tungstic Hæmatoxylin** (*Journ. Exp. Med.*, v, 1900, p. 19; *Zeit. wiss. Mik.*, xviii, 1901, p. 178):

Hæmatoxylin	0·1
Water	80·0
10 per cent. solution of (MERCK'S) phospho-tungstic acid	20·0
Peroxide of hydrogen (U.S. Ph.)	0·2

(Dissolve the hæmatoxylin, add the acid, then the peroxide.) Stain sections two to twenty-four hours, wash out with water.

A *polychromic* stain, nuclei blue, intercellular substances pink. I consider this a fine stain.

273. Donaggio's **Tin Hæmatoxylin** (*Ann. Nevrol. Napoli*, xxii, 1904, p. 192).—A 1 per cent. solution of hæmatoxylin is poured slowly into an equal volume of 20 per cent. solution of pink-salt (ammonio-chloride of tin). Keep in the dark.

274. **Osmium Hæmatoxylin**.—Schultze (*Zeit. wiss. Mik.*, xxvii, 1910, p. 465) treats tissues for twenty-four hours or more with osmic acid of 1 per cent., washes well with water, and puts for a couple of days into ripened 0·5 per cent. solution of hæmatoxylin in alcohol of 35 to 50 per cent. Wash out for a day or more with alcohol of 70 per cent. Intense plasma stain.

CHAPTER XIV.

NUCLEAR STAINS WITH COAL-TAR DYES.

275. Introduction.—Very few coal-tar dyes give a precise nuclear or chromatin stain by the *progressive* method (§ 209). Two of them—methyl green and Bismarck brown—are pre-eminently progressive chromatin stains. Many of the others—for instance, safranin, gentian, and especially dahlia—may be made to give a progressive nuclear stain with fresh tissues by combining them with acetic acid; but in general are not so suitable for this kind of work as the two colours first-named.

Again, very few coal-tar dyes give a pure plasmatic stain (one leaving nuclei unaffected). The majority give a diffuse stain, which in some few cases becomes by the application of the *regressive* method (§ 209) a most precise and splendid chromatin stain.

But plasma staining is generally done by the progressive method.

The basic anilin dyes were at one time greatly in vogue for the staining of chromatin in researches on the structure of nuclei. They have been little used for that purpose since the working out of the iron hæmatoxylin process, which gives a more energetic stain. But they may still be useful as a means of controlling the iron hæmatoxylin process, which frequently stains all sorts of things besides chromatin, which does not occur with the best tar colour stains.

The acid and neutral anilin dyes afford some of our best plasma stains.

I recommend—for staining nuclei of fresh tissues, methyl green; for staining nuclei of fixed tissues by the regressive method, safranin for a red stain; and gentian violet or Thionin for a blue one; as a plasma stain for sections, Säurefuchsin; for entire objects picric acid.

A. *Progressive Stains.*

276. Methyl Green.—This is the most common in commerce of the "anilin" greens. It appears to go by the synonyms of *Methylanilin green, Grünpulver, Vert Lumière, Lichtgrün*; these two last are in reality the name of another colour. When first studied by Calberla, in 1874 (*Morphol. Jahrb.*, iii, 1887, p. 625), it went by the name of *Vert en cristaux.* It is commonly met with in commerce under the name of more costly greens, especially under that of iodine green. It is important not to confuse it with the latter, nor with aldehyde green (*Vert d'Eusèbe*), nor with the phenylated rosanilins, *Paris green*, and *Vert d'Alcali*, or *Véridine.*

Methyl green is the chloromethylate of zinc and penta-methyl-rosanilin-violet. It is obtained by the action of methyl chloride on methyl violet. The commercial dye always contains unconverted methyl violet as a consequence of defective purification. It is sometimes adulterated with anilin blue (soluble blue). It is also sometimes adulterated with a green bye-product of the manufacture—the chloride of nona-methyl-para-leukanilin (see BENEDIKT and KNECHT'S *Chemistry of the Coal-tar Colours*). For tests for purity see MAYER, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 312, and FISCHER, *Fixirung, Färbung, u. Bau des Protoplasmas*, p. 89.

Methyl green is extremely sensitive to alkalies. It is therefore important to use it only in *acidified* solutions and to use only acid, or at least perfectly neutral fluids for washing and mounting.

This is an *extremely important histological reagent.* Its chief use is as a *chromatin* stain for *fresh, unfixed tissues.* For this purpose it should be used in the form of a strong aqueous solution containing a little acetic acid (about 1 per cent. in general). *The solutions must always be acid.* If the tissues have been previously fixed with acetic acid you will not get a chromatin stain. The same applies to fixation with acetic acid sublimate: whilst pure sublimate will allow of a chromatin stain (BURCKHARDT, *La Cellule*, xii, 1897, p. 364). You may wash out with water (best acidulated) and mount in some acid aqueous medium containing a little of the methyl green in solution. The mounting medium, if aqueous, *must be acidulated.*

Employed in this way, with *fresh unfixed* tissues, methyl green is a pure chromatin stain, in the sense of being a precise colour reagent for chromatin. For *in the nucleus* it stains nothing but chromosomes or chromatin elements; it does not stain plasmatic nucleoli (unless indeed these contain chromatin), nor caryoplasm, nor achromatic filaments. *Outside* the nucleus it stains some kinds of cytoplasm and some kinds of formed material, especially glandular secretions (silk for instance, and mucin). The chromatin elements are invariably stained of a bright green (with the exception of the nuclein of the head of some spermatozoa), whilst extra-nuclear structures are in general stained in tones of blue or violet. But this metachromatic reaction is probably due to the methyl-violet impurity, and is not obtained with a chemically pure methyl green.

Staining is *instantaneous*; overstaining never occurs. The solution is very penetrating, kills cells instantly without swelling or other change of form, and preserves their forms for at least some hours, so that it may be considered as a delicate fixative. It may be *combined without precipitating* with divers fixing or preserving agents. Osmic acid (of 0·1 to 1 per cent.) may be added to it, or it may be combined with solution of Ripart and Petit (this is an excellent medium for washing out in and mounting in).

Alcoholic solutions may also be used for staining. They also *should be acidulated with acetic acid.*

The stain does not keep easily. It is difficult to mount it satisfactorily in balsam, because the colour does not resist alcohol sufficiently (unless this be charged with the colour). The resistance of the colour to alcohol is, however (at all events if it be used in the Ehrlich-Biondi combination), considerably increased by treating the sections for a few minutes with tincture of iodine before staining (M. Heidenhain).

Of preparations mounted with excess of colour in the usual aqueous media, I find the most fortunate only survive for a few months. Dr. Henneguy, however, writes to me that it keeps well in Brun's glucose medium.

It was first pointed out, I believe, by Heschl (*Wiener med. Wochenschr.*, 2, 1879), that methyl green is a reagent for amyloid degeneration. His observations were confirmed by Curschmann (*Virchow's Arch.*, vol. lxxix, 1880, p. 556), who showed that it colours amyloid substance of an intense violet; but this, as pointed out by SQUIRE (*Methods and Formulæ*, etc., Churchill, 1892, p. 37), is undoubtedly due to its containing methyl violet as an impurity.

277. Bismarck Brown (Manchester Brown, Phenylen Brown, Vesuvin, La Phénicienne).—A fairly pure nuclear stain that will work either with fresh tissues or with such as have been hardened in chromic acid, or otherwise.

The colour is not very easily soluble in water. You may boil it in water, and filter after a day or two (WEIGERT, in *Arch. mik. Anat.*, xv, 1878, p. 258). You may add a little acetic or osmic acid to the solution. MAYSEL (*ibid.*, xviii, 1880, pp. 237, 250) dissolves the colour in acetic acid (this solution does not give a permanent stain). Alcoholic solutions may also be used, *e.g.* saturated aqueous solution diluted with one third volume of 90 per cent. alcoholic; or CALBERLA's glycerin-and-alcohol mixture, or dilute glycerin (say of 40 per cent. to 50 per cent.) may very advantageously be employed.

The watery solutions must be frequently filtered (but then much of the colour is retained on the filter). The addition to them of carbolic acid has been recommended (vide *Journ. Roy. Mic. Soc.*, 1886, p. 908). Bismarck brown stains rapidly, but never overstains. The stain is permanent both in balsam and in glycerin.

This colour may be used as a chromatin stain for fresh tissues in the same way as methyl green. HERLA (*Arch. Biol.*, xiii, 1893, p. 423) employs for ova of *Ascaris* a mixture of 0·25 parts vesuvin, 0·25 malachite green, 10 of glycerin and 100 of water, and washes out with weak glycerin.

The chief use of this colour is for progressive staining; but it may be employed for staining by the regressive method (see § 289), and also for *intra-vitam* staining (§ 208) (for this purpose it is necessary to see that the colour employed be pure *and neutral*).

278. Methyl Violet (Methylanilin Violet, Anilin Violet, Paris Violet).—GRASER (*Deutsche Zeit. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. wiss. Mik.*, v, 3, 1888, p. 378) stains sections from twelve to twenty-four hours in a solution so dilute that at the end of that time the sections will have taken up all the colour from the liquid. They are then washed out for a short time in acidulated alcohol, and then in pure alcohol. The method is applicable to objects fixed in Flemming's mixture.

279. Other Progressive Stains.—Most of the basic tar colours used for regressive staining will also give by the progressive method a nuclear stain of greater or less purity if used in solutions acidified with acetic acid. Amongst these may be mentioned *thionin*, which need not even be acidified; also, for fresh tissues especially, *gentian violet, dahlia*, and *toluidin blue.*

B. *Regressive Stains.*

280. The Practice of Regressive Staining : The Staining Bath.—*Sections only,* or material that is thin enough to behave like sections, such as some membranes, can be stained by this method.

The solutions employed are made with alcohol, water, or anilin, or sometimes other menstrua, according to the solubility of the colour. There seems to be no special object in making them with alcohol if water will suffice, the great object being to get *as strong a solution as possible.* Indeed, the solutions made with strong alcohol are found not to give quite such good results as those made with water or weak alcohol. Alcohol of 50 per cent. strength, however, may be said to constitute a very generally desirable medium. The sections must be *very thoroughly* stained in the solution. As a general rule they cannot be left too long in the staining fluid. With the powerful solutions obtained with anilin a few minutes or half an hour will usually suffice, but to be on the safe side it is frequently well to leave the sections twelve to twenty-four hours in the fluid. Up to a certain point the more the tissues are stained the better do they resist the washing-out process, which is an advantage. Some workers, indeed, prefer weak solutions ; so HEIDENHAIN, *Encycl. mik. Technik,* i, pp. 433, 434 ; but the nature of the fixing agent should be taken into account.

Material fixed in chromic or chromo-osmic mixtures gives a *sharper and more selective* stain than material fixed in sublimate or the like. In fact, to *ensure the best results, only material fixed in chromic mixtures* (or Hermann's fluid) *should be employed.*

During the staining the tissues become *overstained,* that is, charged with colour in an excessive and diffuse manner. The stain must now be *differentiated* by removal of the excess of colour.

281. Differentiation.—This is generally done with alcohol, sometimes *neutral,* sometimes *acidulated* (with HCl). The stained sections, if loose (celloidin sections), are brought into a watch-glassful of alcohol ; if mounted in series on a slide they are brought into a tube of alcohol (differentiation *can* be done by simply pouring alcohol on to the slide, but it is better to use a tube or other bath). It is in either case well to *just rinse* the sections in water, or even to wash them well in it, before bringing them into alcohol.

The sections in the watch-glass are seen to give up their colour to the alcohol in clouds, which are at first very rapidly formed, afterwards more slowly. The sections on the slide are seen, if the slide be gently lifted above the surface of the alcohol, to be giving off their colour in the shape of rivers running down the glass. In a short time the formation of the clouds or of the rivers is seen to be *on the point of ceasing* ; the sections have become *pale* and somewhat *transparent,* and (in the case of chrom-osmium objects) have *changed colour,* owing to the coming into view of the general ground colour of the tissues. (Thus chrom-osmium-safranin sections turn from an opaque red to a delicate purple.) At this point the differentiation is complete, or nearly so.

It is generally directed that absolute alcohol be taken for differentiation. This may be well in some cases, but in general 95 per cent. is found to answer perfectly well. HEIDENHAIN (*Encycl.,* i, p. 434) takes *methyl* alcohol.

The hydrochloric-acid-alcohol extracts the colour *much more quickly from resting nuclei than from kinetic nuclei.* Therefore, washing out should be done with neutral alcohol whenever it is desired to have resting nuclei stained as well as dividing nuclei; the acid process serving chiefly to *differentiate karyokinetic figures.*

The proportion of HCl with which the alcohol should be acidified for the acid process should be about 1 : 1000, or less; seldom more.

The length of time necessary for differentiating to the precise degree required varies considerably with the nature of the tissues and the details of the process employed ; all that can be said is that it generally lies between thirty seconds and two minutes. The acid process is *vastly more rapid* than the neutral process, and therefore of course more risky.

There exists also a method of differentiation known as *substitution*—one stain being made to wash out another. Thus methylen blue and gentian violet are discharged from tissues by aqueous solution of vesuvin or of eosin; fuchsin is discharged from tissues by aqueous solution of methylen blue. The second stain "substitutes" itself for the first in the general "ground" of the tissues, leaving, if the operation has been successfully carried out, the nuclei stained with the first stain, the second forming a "contrast" stain. In the paper of RESEGOTTI in *Zeit. wiss. Mik.*, v, 1888, p. 320, it is stated as a very general rule that colours that do *not* give a nuclear stain by the regressive method will wash out those that *do*. But RESEGOTTI used the second colour in *alcoholic* solution; so that it remains uncertain how far the differentiation should be attributed to the second colour itself, and how far to the alcohol used as a vehicle. The same remark applies to BENDA's Safranin-and-Lichtgrün process.

282. Clearing.—After due differentiation, the extraction of the colour may be stopped by putting the sections into water; but the general practice is to clear and mount them at once.

You may clear with clove oil or anilin, *which will extract some more colour* from the tissues. Or you may clear with an agent that does not attack the stain (cedar oil, bergamot oil, xylol, toluol, etc.; see the chapter on Clearing Agents). If you have used neutral alcohol for washing out, you had perhaps better clear with clove oil, as neutral alcohol does not always, if the staining have been very prolonged, extract the colour perfectly from extra-nuclear parts. But if you have not stained very long, and if you have used acidulated alcohol for washing out, clove oil is not necessary, and it may be better not to use it, as it somewhat impairs the brilliancy of the stain. A special property of clove oil is that it helps to differentiate karyokinetic figures, as it *decolours resting nuclei more rapidly than those in division.*

Some colours are much more sensitive to the action of clove oil than others; and much depends on the quality of this much-adulterated essence. New clove oil extracts the colour more quickly than old; and aniline than clove oil.

Series of sections on slides are conveniently cleared by pouring the clearing agent over them.

After clearing you may either mount at once in damar or balsam; or, stop the extraction of the colour, if clove oil have been used, by putting the sections into some medium that does not effect the stain (xylol, cedar oil, etc.). Chloroform should be avoided, either as a clearer or as the menstruum for the mounting medium.

283. General Results.—The results depend in great measure on the previous treatment of the tissues. If you have given them a prolonged fixation in Flemming's *strong* chromo-aceto-osmic mixture, and have differentiated after staining with acid alcohol and cleared with clove oil, you will get, with some special exceptions, nothing stained but nucleoli and the chromatin of *dividing* nuclei, that of resting nuclei remaining unstained. If you have given a lighter fixation, with Flemming's weak mixture or some other fixing agent not specially inimical to staining, and have differentiated after staining with neutral alcohol, you will get the chromatin of *resting* nuclei stained as well. Either process may also stain mucin, the ground-substance of connective tissues (especially cartilage), the bodies of Nissl in nerve-cells, and the yolk of ova.

284. HENNEGUY'S Permanganate Method (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 397).—Sections are treated for five minutes with 1 per cent. solution of permanganate of potassium. They are then washed with water and stained (for about half the time that would have been taken if they had not been mordanted with the permanganate) in safranin, rubin, gentian violet, vesuvin, or the like, and are differentiated with alcohol, followed by clove oil in the usual way.

The mordanting action of the permanganate is so energetic that if it has been overmuch prolonged before staining with safranin, or, still more, with rubin, it becomes almost impossible to differentiate the sections properly; it may be necessary to leave them for a month or more in clove oil.

285. OHLMACHER'S Formaldehyde Process (*Medical News*, February 16th, 1895).—Ohlmacher states that formaldehyde is a powerful mordant for tar colours. Tissues may either be mordanted separately by treatment for a short time (one minute is enough for cover-glass preparations) with a 2 per cent. to 4 per cent. formalin solution; or the formalin may be combined with the stain. One gramme of fuchsin or methylen blue dissolved in 10 c.c. of absolute alcohol may be added to 100 c.c. of 4 per cent. formalin solution. Sections are said to stain in half a minute and to resist alcohol much more than is the case with those treated by the usual solutions.

286. Safranin.—One of the most important of these stains, on account of its power, brilliancy, and permanence in balsam, and the divers degrees of electivity that it displays for the nuclei and other constituent elements of different tissues.

The great secret of staining with safranin is *to get a good safranin.* In ordering it, from Grübler & Hollborn or elsewhere, it is well to specify whether you want it for staining nuclei or for staining elastic fibres, or for what other purpose you may require it. There are presumably at least a score of sorts of safranin in the market, differing to a considerable extent in colour, weight, solubility, and histological action. Some are easily soluble in water and not so in alcohol, some the reverse, and some freely soluble in both. The brand I have been using for a long time, which gives good results, is the "Safranin O" of Grübler & Co.

Staining.—The majority of safranins are not sufficiently soluble in water, so that solutions in other menstrua must be employed.

Pfitzner (*Morph. Jahrb.*, vi, p. 478, and vii, p. 291) advised a solution of safranin 1 part, absolute alcohol 100 parts, and water 200 parts, the last to be added only after a few days.

Flemming (*Arch. mik. Anat.*, xix, 1881, p. 317) used a concentrated solution in absolute alcohol, diluted with about one half of water.

Babes (*ibid.*, 1883, p. 356) used (A) a mixture of equal parts of concentrated alcoholic solution and concentrated aqueous solution (this is very much to be recommended), or (B) a concentrated or supersaturated aqueous solution made with the aid of heat.

Some people still employ simple aqueous solutions.

The anilin solution of Babes (*Zeit. wiss. Mik.*, iv, 1887, p. 470) consists of water 100 parts, anilin oil 2 parts, and an excess of safranin. The mixture should be warmed to from 60° to 80° C., and filtered through a wet filter. This solution will keep for a month or two.

Zwaardemaker (*ibid.*, iv, 1887, p. 212) makes a mixture of about equal parts of alcoholic safranin solution and anilin water (saturated solution of anilin oil in water;—to make it, shake up anilin oil with water, and filter). This, I find, will keep for many months, perhaps indefinitely.

I myself use equal parts of saturated solution in anilin water, and saturated solution in absolute alcohol.

Differentiation.—For general directions see §§ 281 and 282.

FLEMMING's *acid* differentiation (*Zeit. wiss. Mik.*, i, 1884, p. 350).—Differentiate, until hardly any more colour comes away, in alcohol acidulated with about 0·5 per cent. of hydrochloric acid, followed by pure alcohol and clove oil. (You may use the HCl in watery solution if you prefer it.) Or you may use a lower strength, viz. 0·1 per cent. at most (see *Arch. mik. Anat.*, xxxvii, 1891, p. 249); and this I find is generally preferable.

Objects are supposed to have been well fixed—twelve hours at least—in the *strong* chromo-aceto-osmic mixture, and stained for some hours. In this way you get kinetic chromatin and nucleoli alone stained.

PODWYSSOZKI (*Beitr. z. Path. Anat.*, i, 1886, p. 289) differentiates (for from a few seconds to two minutes) in a strongly alcoholic solution of picric acid, followed by pure alcohol. Same results (except that the stain will be brownish instead of pure red).

BABES recommends treatment with iodine, according to the method of GRAM (see next section). This process has also been recommended by PRENANT (*Int. Monatsschr. Anat.*, etc., iv, 1887, p. 368).

It has been shown by OHLMACHER (*Journ. Amer. Med. Assoc.*, vol. xx, No. 5, February 4th, 1893, p. 111) that if tissues be treated with iodine or picric acid after staining with safranin, there may be produced in the tissue elements a precipitate of a dark red substance of a crystalline nature, but of lanceolate, semilunar, falciform, or navicellar forms. The precipitate is formed both in normal and pathological tissue, readily in carcinomatous tissues; and Ohlmacher concludes that many of the bodies that have been described as "coccidia," "sporozoa," or other "parasites" of carcinoma are nothing but particles of this precipitate.

See also the differentiation process of MARTINOTTI and RESEGOTTI (*Zeit. wiss. Mik.*, iv, 1887, p. 328) for alcohol-fixed material; and of GARBINI (*Zeit. wiss. Mik.*, v, 2, 1888, p. 170).

In preparations made with chromo-aceto-osmic acid, safranin stains, besides nuclei, elastic fibres, the cell bodies of certain horny epithelia, and the contents of certain gland-cells (mucin, under certain imperfectly ascertained conditions).

The stain is perfectly permanent.

287. **Gentian Violet** may be used in aqueous solution, or as directed for safranin.

In some cases it may be useful to employ the method devised by GRAM for the differentiation of bacteria in tissues (*Fortschr. d. Medicin.*, ii, 1884, No. 6; *British Med. Journ.*, Sept. 6th, 1884, p. 486; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 817). In this the sections are treated, after staining, with a solution composed of—

Iodine	1 gramme,
Iodide of potassium . . .	2 grammes,
Water	300 ,,

for two or three minutes, until they become black. They are then differentiated with neutral alcohol, until they turn grey, and are then finally differentiated with clove oil.

By this process, in resting nuclei the nucleoli alone are stained, or the chromatin if stained is pale; in dividing nuclei the chromatin is stained with great intensity, being nearly black in the equatorial stage.

Gentian violet is an exceedingly powerful stain, quite as precise as safranin.

The stain keeps well. It is more or less dichroic, possibly owing to the fact that the dye is not a pure substance, but a mixture of "Krystallviolett" and methyl violet.

HERMANN (*Arch. mik. Anat.*, xxxiv, 1889, p. 58) first stains for twenty-four hours or more in safranin, differentiates incompletely with alcohol, then stains for three to five minutes in the anilin-water gentian solution, treats with the iodine solution for one to three hours, and finally differentiates with absolute alcohol.

288. **Thionin.**—The hydrochloride of thionin, or violet of Lauth, is a colour chemically nearly allied to methylen blue. Its action is so selective from the first that it may almost be considered to be a progressive stain. If you stain for only a short time (a few minutes) in a concentrated aqueous solution, hardly anything but the chromatin will be found to be stained. If the staining be prolonged, plasmatic elements will begin to take up the colour. After a short stain no special differentiation is required; all that is necessary is to rinse with water, dehydrate, and mount. After a strong stain you differentiate with alcohol in the usual way, with this advantage, that the stain is so highly resistant to alcohol that there is no risk whatever of overshooting the mark; the stain will not be more extracted in

an hour than that of gentian or dahlia is in a minute, so that the process may be controlled under the microscope if desired. For this reason I think this stain may be useful to beginners, but I myself prefer gentian. It is a very powerful stain.

Thionin is a specific stain for mucin, *q. v.* Some observers have found the stain to fade. Wolff (*Zeit. wiss. Mik.*, xv, 1899, p. 312) says that to avoid this, preparations should be mounted in a little solid colophonium or balsam melted over a flame. Felizat and Branca (*Journ. Anat Phys.*, xxxiv, 1898, p. 590) mount without a cover. Henneguy (*in litt.*) clears with acetone.

King (*Anat. Record*, iv, 1910, p. 236) stains with a saturated solution in carbolic acid of 1 per cent., and finds the stain permanent.

Nicolle's "thionine phèniquée" consists of 1 part of saturated solution in alcohol of 50 per cent., and 5 parts of 2 per cent. aqueous solution of carbolic acid.

289. Other Regressive Stains.—The following may be useful:—

Dahlia, according to Flemming (*Arch. mik. Anat.*, xix, 1881, p. 317), best used in aqueous solution, either neutral or acidified with acetic acid, and differentiated with neutral alcohol. A pure blue stain, which keeps well. See also Schuberg, in *Zeit. wiss. Zool.*, lxxiv, 1903, p. 7, and lxxxvii, 1907, p. 557.

Victoria Blue (Victoriablau) (Lustgarten, *Med. Jahrb. k. Ges. d. Aerzte zu Wien*, 1886, pp. 285—291).—This dye ("Victoriablau 4 A") has a special affinity for *elastic fibres.* For this object Lustgarten recommends an alcoholic solution of the dye diluted with two to four parts of water. Fixation in chrom-osmium, or at least in a chromic mixture, is, I believe, a necessary condition to this reaction. And you must stain for a long time.

Victoria has also a special affinity for *mucus-cells,* from which it is not washed out by alcohol, and for cartilage.

This stain keeps very well.

With **Toluidin Blue** I have had some superb stains of chromatin, unfortunately accompanied by a diffuse staining of cytoplasm.

Mann (*Zeit. wiss. Mik.*, xi, 1894, p. 489) states that he has had good results by staining with it after eosin.

See further as to the micro-chemical properties of this dye. Harris, *The Philadelphia Medical Journal*, May 14th, 1898. It much resembles methylen blue.

Metzner (Nagel's *Handb. Phys.*, ii, 1907, p. 915) mordants sections, before staining, for three quarters of an hour in iron alum.

Magdala Red (Naphthalin Red, Rose de Naphthaline).

Fuchsin (meaning the basic fuchsins, a series of Rosanilin salts having very similar reactions, and found in commerce under the names of Fuchsin, Anilin Red, Rubin, Rosein, Magenta, Solferino, Corallin).—Graser (*Deutsche Zeit. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. wiss. Mik.*, v, 1888, p. 378) stains for twelve to twenty-four hours in a dilute aqueous solution, washes out for a short time in alcohol, stains for a few minutes in aqueous solution of methylen blue, and dehydrates with alcohol. A double stain. Chromatin and nucleoli red, all the rest blue.

Ziehl's **Carbolic Fuchsin** (*Zeit. wiss. Mik.*, vii, 1890, p. 39) consists of fuchsin 1 grm., acid. carbol. crist. 5 grms., alcohol 10 grms., aq. dest. 100 grms. The stain is differentiated with alcohol followed by clove oil.

Kresofuchsin (Röthig, *Arch. mik. Anat.*, lvi, 1900, p. 354).—Its aqueous solution is red, and stains mucus, cartilage, keratin, and nuclei red, whilst its alcoholic solution is blue and stains elastin blue. See also under "Connective tissues."

Bismarck Brown has this advantage, that being sufficiently resistant to alcohol it may be utilised for staining *entire objects.*

Kaiser (*Biblioth. Zool.*, H. 7, 1 Hälft, 1891; *Zeit. wiss. Mik.*, viii, 1891, p. 363) stains for forty-eight hours, and at a temperature of 60° C. in saturated solution of Bismarck brown in 60 per cent. alcohol (the solution to be made in boiling alcohol), and washes out (until all is decoloured except the karyokinetic figures) in 60 per cent. alcohol, containing 2 per cent. hydrochloric acid or 3 per cent. acetic acid.

Methyl Violet. See *ante*, § 278

Benzoazurin (Martin, *Zeit. wiss. Mik.*, vi, 3, 1889, p. 193). Stain for an hour or so in dilute aqueous solution, and wash out with HCl alcohol.

Methylen Blue.

Nigrosin (Errera, *Proc.-Verb. Soc. Belge de Mik.*, 1881, p. 134) gives a good stain which resists alcohol well.

Methyl Green is sometimes useful in certain mixtures (see next chapter).

CHAPTER XV.

PLASMA STAINS[*] WITH COAL-TAR DYES.

290. Introduction.—By a plasma stain is meant one that stains the extra-nuclear parts of cells and the formed material of tissues, or one of these.

The plasma stains described in this chapter are for the most part those obtained by means of "acid" dyes (§ 203); but some of them are obtained by means of "neutral" dyes (§ 203), and a few by "basic" dyes.

The mode of staining is generally progressive, almost always so when acid colours, used substantively (§ 205), are employed. But the regressive method, with differentiation, is sometimes made use of, especially when a mordant has been used with the dye.

In some processes, *e. g.*, Flemming's orange method, a basic and an acid dye (or *vice versâ*) being employed *in succession*, there is formed *in the tissues* a neutral colour (§ 203) which effects the desired stain. These may be considered as adjective stains, the first colour serving as a mordant for the second. Not any two dyes taken at haphazard will behave in this way: they must be such as to form by combination a *suitable* neutral lake (cf. § 203). The basic dye may be made the primary stain, as in Flemming's process: or the contrary.

In such stains as Reinke's orange method, or the Ehrlich-Biondi mixture, and many others, one or more neutral colours are formed *in the mixture* and stain progressively.

I am not acquainted with any plasma stain that is thoroughly satisfactory for delicate work. I recommend for sections Säurefuchsin, either alone or in the form of Ehrlich-Biondi mixture, or Ehrlich's triacid: for material in bulk, picric acid (but only for rough work).

---

[*] This chapter includes only such stains as are used in *ordinary* work on tissues in bulk or sections, stains for *special* purposes being treated under "Nervous tissue," "Blood," etc. It includes some double or triple stains that affect nuclei as well as plasma, but in different hues.

291. Säurefuchsin (Acid Fuchsin, Fuchsin S, Acid Rubin, Rubin S, Saurerubin, Acid Magenta, Magenta S).—The chemical description of this acid colour has been given (§ 203): it must not be confounded with basic fuchsin, as seems to have been done by some writers.

This dye is highly soluble in water, less so in alcohol. I use a 0 5 per cent. solution in water and allow it to act on sections for a few minutes in the case of easily stainable material, or twenty-four hours or more for chrom-osmium material. The stain is fast to neutral alcohol. It is very sensitive to alkalies, so that overstains can easily be removed by washing for a few minutes in tap-water. Acids strengthen the stain, so that it is frequently useful to treat sections after staining for a few seconds with acidulated water. A good stain should show the reticulum of cytoplasm, together with nuclear spindles and asters, stained red, and connective tissue strongly brought out. It may be advisable to acidify the staining bath *very slightly*. Successful stains are admirably sharp.

292. Pyronin.—A basic dye, red, only used (as far as I can find) in mixtures. PAPPENHEIM (*Arch. Path. Anat.*, clxvi, 1901, p. 427) takes two parts 1 per cent. solution of methyl green and one part 1 per cent. solution of pyronin, stains sections for five minutes, rinses, and differentiates in a solution of resorcin or hydroquinon in absolute alcohol. According to CORTI and FERRARA, *Mon. zool. Ital.*, xvi, 1905, p. 319, this mixture generally stains chromatin green and cytoplasm red, but in Flemming or Hermann material the reverse. It seems to me a *coarse* plasma stain, but likely to be sometimes useful.

UNNA'S CARBOL-PYRONIN-METHYL GREEN modification (*Encycl. Mik. Techn.*, 1910, ii, p. 412: I am indebted for the formula to Dr. GAUDLITZ) is as follows: Stain for five to ten minutes at 30° to 40° C. in methyl green 0·15 parts, pyronin 0·25, alcohol 2·5, glycerin 20, and carbolic acid of 0·5 per cent. to make up 100 volumes. Cool rapidly, rinse, dehydrate, and pass through bergamot oil, or xylol or benzol (*not clove-oil*) into balsam. Brings out bacteria (rod) in organic liquids. The mixture may be had from Grübler & Hollborn.

293. **Orange G.**—This is the benzenazo-beta-naphthol-disulphonate of soda (to be obtained from Grübler & Hollborn, and not to be confounded with about a dozen other colours that are on the market under the name of "Orange," with or without a suffix). As indicated by its chemical description, this is an "acid" colour.

It is easily soluble in water, less so in alcohol. Use as directed for Säurefuchsin. Almost, if not quite, as precise a stain as Säurefuchsin. It does not overstain, but may wash out other dyes.

294. **Säurefuchsin and Orange G.**—I have had good results by mixing the aqueous solutions of these two dyes, but unfortunately have not noted the proportions. SQUIRE (*Methods and Formulæ*, p. 42) takes 1 grm. Säurefuchsin, 6 grms. Orange G. in 60 c.c. of alcohol, and 240 c.c. of water. See also under "connective tissues."

295. EHRLICH-BIONDI **Mixture** (or EHRLICH-BIONDI-HEIDENHAIN **Mixture**) (*Pflüger's Arch.*, xliii, 1888, p. 40).

To 100 c.c. saturated aqueous solution of orange add with continual agitation 20 c.c. saturated aqueous solution of Säurefuchsin (Acid Fuchsin) and 50 c.c. of a like solution of methyl green.

(According to Krause [*Arch. mik. Anat.*, xlii, 1893, p. 59], 100 parts of water will dissolve about 20 of Säurefuchsin [Rubin S], 8 of orange G. and 8 of methyl green.) The solutions must be *absolutely saturated*, which only happens after several days.

Dilute the mixture with 60 to 100 volumes of water. The dilute solution ought to redden if acetic acid be added to it; and if a drop be placed on blotting-paper it should form a spot bluish green in the centre, orange at the periphery. If the orange zone is surrounded by a broader red zone, the mixture contains too much fuchsin.

According to M. HEIDENHAIN ("Ueber Kern u. Protoplasma," in *Festschr. f. Kölliker*, 1892, p. 115) the orange to be used should be "Orange G"; the Acid Fuchsin or Säurefuchsin should be "Rubin S" ("Rubin" is a synonym of Fuchsin) and the methyl green should be "Methylgrün OO." And it is *absolutely necessary* that these ingredients be those prepared under those names by the *Actienfabrik für Anilinfabrikation in Berlin.* They can be obtained from Grübler & Hollborn, either separately, or as a mixture of the three dyes in powder (which I do not recommend).

The strong solutions directed to be taken readily precipitate on being mixed. To avoid this it is recommended by SQUIRE (*Methods and Formulæ*, etc., p. 37) to dilute them before mixing.

Other proportions for the mixture have been recommended by KRAUSE (*loc. cit. supra*), viz. 4 c.c. of the Säurefuchsin solution, 7 of the orange G and 8 of the methyl green; the mixture to be diluted 50 to 100-fold with water. THOMÉ (*Arch. mik. Anat.*, lii, 1898, p. 820) gives the proportions 2 : 5 : 8, and dilutes 100-fold.

Stain sections (N.B. *sections only*) for six to twenty-four hours. Dehydrate with alcohol, clear with xylol, and mount in xylol balsam.

In the intention of the observers who have elaborated this stain it is a *progressive* stain, and *not* a regressive one. It does not require any differentiation, and the sections should be got through the alcohol into xylol as quickly as possible in order to avoid any extraction of the methyl green, which easily comes away in the alcohol. DRÜNER (*Jena. Zeit.*, xxix, 1894, p. 276) stains for ten minutes in the *concentrated* solution, treats for one minute with alcohol containing 0·1 per cent. of hydrochloric acid, and then with neutral alcohol.

The best results are obtained with *sublimate material*; chrom-osmium material, and the like, give a much inferior stain. Preparations made with the usual mixture, as given above, are liable to fade; by *acidifying* the mixture a stronger and more sharply selective stain is obtained, which does not fade. But too much acid must not be added, as this would cause a staining of the interfilar substances. According to the *Encycl. mik. Technik*, you may add 15 to 24 drops of 0·2 per cent. acetic acid to 100 c.c. of the diluted solution.

Another process of acidification is given by M. HEIDENHAIN (*Ueber

Kern und Protoplasma, p. 116); for this see *fourth edition*. See also ISRAEL (*Praktikum Path. Hist.*, 2 Aufl., Berlin, 1893, p. 69); TRAMBUSTI (*Ricerche Lab. Anat. Roma*, v, 1896, p. 82; *Zeit. wiss. Mik.*, xiii, 1896, p. 357): and THOMÉ (*op. cit. supra*). EISEN (*Proc. Calif. Acad.* [3], i, 1897, p. 8) acidifies with oxalic acid.

After acidification the solution must not be filtered, and if it has been kept for some time a little more acid must be added.

Before staining (M. HEIDENHAIN, *loc. cit.*), sections should be treated for a couple of hours with 0·1 per cent. acetic acid, then for ten to fifteen minutes with officinal tincture of iodine, and be rinsed with alcohol before bringing into the stain. The treatment with acid is necessary in order to ensure having the sections *acid* on mounting in balsam. The primary object of the iodine is to remove any sublimate from the preparations, but it also is said to enhance the power of staining of the chromatin with methyl green, and to produce a more selective staining of protoplasmic elements.

The stain is a very fine one when successful. But it is *very capricious*. The correct result should be a precise chromatin stain combined with a precise stain of the plastin element of cytoplasm by the Säurefuchsin. Now the least defect or excess of acidity causes the plasma stain of the Säurefuchsin to become a diffuse one, instead of being sharply limited to the plastin element. It is difficult to dehydrate the sections without losing the methyl green. For this reason the stain will only work with very thin sections: to be quite sure of good results, the sections should be of not more than 3 μ in thickness, and if they are over 5 the desired results are almost hopeless. The stain keeps very badly. I admit that the method has its *raison d'être* for the very special objects for which it was imagined —for the researches on cell-granulations for which EHRLICH employed the three colours, or for the researches on the plastin element of cytoplasm for which MARTIN HEIDENHAIN employed the mixture; for the study of gland cells; and for similar objects. But to recommend it, as has been done, as a general stain for ordinary work, is nothing but mischievous exaggeration. For it is far from having the qualities that should be possessed by a normal section stain.

Workers have at length found this out, and it is now but little used except for the special purposes above indicated.

296. Ehrlich's **"Triacid" Mixture.**—This name would seem to indicate that the mixture contains three "acid" colours, which is not the case, methyl green being a strongly "basic" colour. Ehrlich explains in a letter to Mayer (see also Ehrlich and Lazarus, *Die Anæmie*, 1898, p. 26) that it is so called "because in it all the three basic groups of the methyl green are combined with the acid dye-stuffs." A very pretty conundrum!

The latest receipt (*op. cit.*, p. 28) is as follows:

Prepare separately saturated solutions of orange G, Säurefuchsin, and methyl green, and let them clarify by settling. Then mix, in the order given, using the same measure-glass, 13 to 14 c.c. of the orange, 6 to 7 of the Säurefuchsin, 15 of distilled water, 15 of alcohol, 12½ of the methyl green, 10 of alcohol, and 10 of glycerin. After adding the methyl green, shake well, but do not filter.

The mixture keeps well. I find its qualities and defects to be much those of the Ehrlich-Biondi mixture. The stain seems more powerful but less delicate, and the methyl green in it appears to have more resistance to alcohol, so that it is better adapted for ordinary work.

Mayer (*Grundzüge*, Lee & Mayer, p. 197) has simplified the formula thus: Take 1 g. methyl green, 2 g. orange, 3 g. Säurefuschin, and dissolve in a mixture of 45 c.c. water, 10 c.c. glycerin, and 20 c.c. alcohol of 90 per cent.

Morel and Doleris (*C. R. Soc. Biol.*, liv, 1902, p. 1255) mix 1 vol. of the solution with one of 8 per cent. formalin and add 0·1 per cent. of acetic acid, and state that thus the methyl green is better fixed in the nuclei.

297. Pianese's **Säurefuchsin-malachite Green** (from Müller, *Arch. Zellforsch.*, viii, 1912, p. 4) consists of 0·5 grm. malachite green, 0·1 grm. Säurefuchsin, and 0·01 grm. Martius yellow in 150 c.c. water and 50 c.c. alcohol. Stain for 24 hours, differentiate with alcohol, containing 1 to 2 drops of HCl per 200 c.c.

298. Picric Acid.—Picric acid gives useful plasma stains after carmine and hæmatoxylin. The *modus operandi* consists merely in adding picric acid to the alcohols employed for dehydrating the objects.

Picric acid has considerable power of washing out other anilin stains; and *in combination with hydrochloric acid it very greatly enhances* the power with which this acid washes out carmine stains. It should, therefore, not be added to the acidulated alcohol taken for differentiating borax-carmine stains, or the like, but only to the neutral alcohol used afterwards. It has the great quality that it can be used for staining *entire objects,* and is much indicated for such objects as small Arthropods or Nematodes, mounted whole.

It can in some cases be employed by dissolving it in the solution of another dye (see Picro-carmine, LEGAL's alum-carmine, § 219, etc.); or (for sections) by dissolving it in the xylol or chloroform used for clearing.

Though picric acid is a useful ground stain, it is at most a *rough* one, being *very diffuse.* It stains, however, horn, chitin, muscle and erythrocytes, with special energy.

According to FRÖHLICH (*Zeit. wiss. Mik.*, xxvii, 1910, p. 349) *picraminic acid* (from Grübler & Hollborn) has some advantages over picric acid.

299. VAN GIESON's **Picro-Säurefuchsin** (from *Zeit. wiss. Mik.*, xiii, 1896, p. 344).—To a saturated aqueous solution of picric acid is added a few drops of saturated aqueous solution of Säurefuchsin, until the mixture has become garnet-red. Or (*Trans. Amer. Micr. Soc.*, xix, 1898, p. 105) to 100 parts of the picric acid solution add 5 parts of 1 per cent. solution of Säurefuchsin. After staining (sections only), rinse with water, dehydrate, and clear in oil of origanum.

OHLMACHER (*Journ. Exper. Med.*, ii, 1897, p. 675) adds 0·5 per cent. of Säurefuchsin to a saturated solution of picric acid which has been diluted with an equal quantity of water. He uses this after previous staining with gentian violet.

RAMÓN Y CAJAL recommends 0·1 grm. of Säurefuchsin to 100 of saturated solution of picric acid (SCHAFFER, *Zeit. wiss. Zool.*, lxvi, 1899, p. 236).

HANSEN (*Anat. Anz.*, xv, 1898, p. 152) adds 5 c.c. of 2 per cent. solution of Säurefuchsin to 100 c.c. saturated solution of picric acid, and for staining adds to 3 c.c. of the mixture one third of a drop of 2 per cent. acetic acid, stains for a few minutes or hours, rinses in 3 c.c. of water with 2 drops of the acidified stain added, dehydrates, clears with xylol, and mounts in xylol-balsam. Connective-tissue red, elastin and all other elements yellow.

WEIGERT (*Zeit. wiss. Mik.*, 1904, p. 3) adds 10 parts of 1 per cent. Säurefuchsin to 100 of saturated picric acid.

See also MÖLLER, *op. cit.*, xv, 1898, p. 172.

This stain is generally used as a contrast stain to follow hæmatoxylin. APÁTHY (*Behrens' Tabellen*, 3rd ed., p. 129) takes for this purpose 1 grm. of Säurefuchsin in 500 c.c. of saturated solution of *picrate of ammonia.*

WILHELMI (*Fauna Flora Golf. Neapel*, xxii, 1909, p. 18) takes 0·2 grm. Säurefuchsin, 0·8 grm. picrate of ammonia, 10 grm. absolute alcohol, and 89 grm. water.

E. and T. SAVINI (*Zeit. wiss. Mik.*, xxvi, 1909, p. 31) use a formula due to BENDA. Ninety-five volumes of saturated solution of picrate of ammonia are mixed with 5 volumes of 1 per cent. solution of Säurefuchsin. For use, two to four drops of saturated solution of picric acid are added to 10 c.c. of the mixture. This neither overstains nor attacks the primary stain.

300. **FLEMMING'S Orange Method** (*Arch. mik. Anat.*, xxxvii, 1891, pp. 249 and 685).—Stain sections *of Flemming or Hermann material* in strong alcoholic safranin solution diluted with anilin water (§ 286); differentiate in absolute alcohol, containing at most 0·1 per cent. of hydrochloric acid, until hardly any more colour comes away; stain for one to three hours in gentian violet (§ 287); wash for a short time in distilled water; treat with concentrated, or at least fairly strong, aqueous solution of orange G. After at most a few minutes, whilst pale violet clouds are still being given off from the sections on agitation, bring them into absolute alcohol until hardly any more colour comes away, clear in clove or bergamot oil, and mount in damar or balsam before the last pale clouds of colour have ceased to come away. The orange *must* be *orange G.*

WINIWARTER and SAINMONT (*Zeit. wiss. Mik.*, xxv, 1908, p. 157, and *Arch. Biol.*, xxiv, 1909, p. 15) stain for 24 hours in the gentian, wash out after the orange for 2 to 3 hours in 100 c.c. absolute alcohol with three to four drops of HCl, and differentiate finally with oil of cloves.

This is not a triple stain in the sense of giving three different colours in the result; it is a nuclear and plasmatic stain in mixed tones; the orange, apparently, combines with the gentian to form a "neutral" dye, soluble in excess of the orange (§ 203) which thus differentiates the stain.

See also FLEMMING in *Arch. Anat. Phys. Anat. Abth.*, 1897, p. 175. Never popular, this clumsy and uncertain process is now little used.

301. REINKE's **Orange Method** (*Arch. mik. Anat.*, xliv, 2, 1894, p. 262). —To a concentrated aqueous solution of gentian violet are added "a few drops" of a like solution of orange G. The solution precipitates in part, owing to the formation of an imperfectly soluble "neutral" colour, but becomes almost clear again if an excess of water be added. The solution is not to be filtered, but the sections are to be stained in the mixture made almost clear by addition of water. It is said that the "neutral" solution may be preserved for future use by adding to it one third of alcohol. After staining (sections previously stained with safranin), you differentiate rapidly with alcohol and clear with clove oil.

I have tried this process and obtained exactly the same results as with Flemming's process, and so have other workers.

ARNOLD'S **Orange Method** (*Arch. Zellforsch.*, iii, 1909, p. 434).—Sections (of chrome material) are treated for five minutes with solution of equal parts of iodine and iodide of potassium in alcohol of 40 per cent., then washed and stained for 4 hours in saturated solution of safranin in alcohol of 75 per cent.: then washed and put for 5 to 15 minutes into solution of seven parts of methylen blue, 0·5 of carbonate of soda and 100 of water, washed, dehydrated, and treated until pale blue with solution of orange G. in oil of cloves. Cytoplasmic reticulum blue on orange ground, nucleoli and centrosomes red. Instead of the safranin, basic fuchsin may be taken.

302. BONNEY'S **Triple Stain** (*Virchow's Arch.*, cxciii, 1908, p. 547, and elsewhere).—Stain sections (of acetic alcohol or sublimate material, not chrome or formol material) for two minutes in a solution of 0·25 parts methyl violet and 1 part pyronin in 100 of water. Wipe slide dry, and flood twice with the following: 2 per cent. aqueous solution of orange G, boiled and filtered, is added drop by drop to 100 c.c. of acetone, with agitation, until there is formed a flocculent precipitate, which redissolves on further addition of the orange. Wash rapidly in pure acetone, and pass through xylol into balsam. Chromatin violet, cytoplasm red, connective tissue yellow, keratin violet. Not adapted for blood films.

303. **Bordeaux R.**—An "acid" dye, giving a general stain taking effect both on chromatin and cytoplasm, and, I consider, a very good plasma stain. I use for chrom-osmium material a 1 per cent. solution, and stain for twelve to twenty-four hours. The stain is sufficiently fast.

304. **Bordeaux R, Thionin, and Methyl Green** (GRÄBERG, *Zeit. wiss. Mik.*, xiii, 4, 1896, p. 460).

305. **Congo Red (Congoroth)** (see GRIESBACH, in *Zeit. wiss. Mik.*, iii, 1866, p. 379).—An "acid" colour. Its solution becomes blue in presence of the least trace of free acid (hence Congo is a valuable reagent for demonstrating the presence of free acid in tissues; see the papers quoted *loc. cit.*). A stain much of the same nature as Säurefuchsin. It is useful for staining some objects during life (see *ante*, § 208). CARNOY (*La Cellule*, xii, 1897, p. 216) has had very good results with it after hæmatoxylin of DELAFIELD. He used 0·5 per cent. solution in water. Note that this colour is not to be confounded with other Congos, as Congo yellow, or brilliant Congo. It is one of the azo dyes.

306. **Congo-Corinth.**—Also an acid dye. HEIDENHAIN (*Zeit. wiss. Mik.*, xx, 1903, p. 179) recommends Congo-Corinth G (or the allied colour **Benzopurpurin 6 B**) (Elberfelder Farbwerke). Sections *must be made alkaline* before staining, by treating them with very weak sal ammoniac or caustic soda, in alcohol. After staining, pass through absolute alcohol into xylol. Used after alum hæmotoxylin, the stain of which it does not cause to fade.

307. **Benzopurpurin.**—According to GRIESBACH (*loc. cit.*, § 305), another "acid" colour very similar in its results to Congo red. See also ZSCHOKKE (*ibid.*, v, 1888, p. 466), who recommends **Benzopurpurin B**, and says that weak aqueous solutions should be used for staining, which is effected in a few minutes, and alcohol for washing out. **Deltapurpurin** may be used in the same way.

See last § as to the necessity of *alkalising* the sections, which Heidenhain states is necessary with all dyes of this group.

308. **Blauschwarz B and Brillantschwarz 3 B** (HEIDENHAIN, *op. cit.*, § 306, p. 183).—Acid azo dyes. To be used in 1 per cent. solution with sections of sublimate material, staining for five to ten minutes. Then stain is a basic dye, such as toluidin blue or safranin.

309. **Neutral Red (Neutralroth)** (EHRLICH, *Allg. med. Zeit.*, 1894, pp. 2, 20; *Zeit. wiss. Mik.*, xi, 1894, p. 250; GALEOTTI, *ibid.*, p. 193).—A "basic" dye. The term "neutral" refers to the hue of its solution. Its neutral red tint is turned bright red by acids, yellow by alkalies. The stain in tissues is in general metachromatic, nuclei being red, cell-bodies yellow (*cf.* ROSIN, in *Deutsche med. Wochenschr.*, xxiv, 1898, p. 615; *Zeit. wiss. Mik.*, xvi, 2, 1899, p. 238). Up to the

present this colour has chiefly been employed for *intra-vitam* staining. Tadpoles kept for a day or two in a solution of 1 : 10,000 or 100,000 absorb so considerable a quantity of the colour that all their tissues appear of a dark red. The stain is limited to cytoplasmic granules (EHRLICH), and to the contents of mucus cells (GALEOTTI).

According to EHRLICH and LAZARUS (*Spec. Pathol. und Therapie*, herausgeg. von NOTHNAGEL, viii, 1, 1898, p. 1; *Zeit. f. wiss. Mik.*, xv, 3, 1899, p. 338) it may be used for *intra-vitam* staining of tissues in the same way as methylen blue, by injection or immersion with contact of air. It is especially a *granule* stain. Similar results are recorded by ARNOLD (*Anat. Anz.*, xvi, 1899, p. 568, and xxi, 1902, p. 418). See also EHRLICH and LAZARUS, *Anæmie*, i, 1898, p. 85; LOISEL (*Journ. de l'Anat. et de la Physiol.*, 1898, pp. 197, 210, 217) (*intra-vitam* staining of sponges); and PROWAZEK (*Zeit. wiss. Zool.*, lxii, 1897, p. 187) (*intra-vitam* staining of Protozoa). I myself have had very good results with it as an *intra-vitam* stain.

According to GOLOVINE (*Zeit. wiss. Mik.*, xix, 1902, p. 176), the stain may be fixed in the tissues by means of sublimate, chromic acid, bichromates, picric acid, or platinum chloride, followed by molybdate of ammonium.

It has also been found useful for staining, in hardened material, the corpuscles of NISSL (*q. v.*) in nerve-cells. S. MAYER (*Lotos*, Prague, 1896, No. 2) states that it also stains degenerating myelin. The solutions that have been employed for staining fixed material are strong aqueous ones, 1 per cent. to concentrated.

310. The Eosins, found in commerce under the names of **Eosin, Saffrosin, Primerose Soluble, Phloxin, Bengal Rose, Erythrosin, Pyrosin B, Rose B, à l'Eau**, etc., are all "acid" phthalein colours. They are not quite identical in their properties. Most of them are soluble both in alcohol and in water, but some only in alcohol ("*Primerose à l'Alcool*").

They are all *diffuse* stains, formerly much used as contrast stains, less so now. HANSEN (*Anat. Hefte*, xxvii, 1905, p. 620) adds 1 drop of acetic acid of 2 per cent. to 9 c.c. of 1 per cent. eosin, which makes the stain more selective.

For **Bengal Rose** see GRIESBACH, *Zool. Anz.*, 1883, p. 172.

Eosin is a specific stain for red blood-corpuscles, and also for certain granules of leucocytes (see under "Blood").

The yolk of some ova takes the stain strongly, so that it is useful in some embryological researches.

311. EHRLICH'S **Indulin-Aurantia-Eosin,** or **Acidophilous Mixture,** or **Mixture C,** or **Mixture for Eosinophilous Cells** (from the formula kindly sent me by Dr. GRÜBLER).—Indulin, aurantia, and eosin, of each two parts; glycerin, thirty parts. This gives a very thick, syrupy solution. To use it, cover-glass preparations may be floated on to it; or sections on slides may have a few drops poured on to them, the slide being laid flat till the stain has taken effect (twenty-four hours for Flemming material). I find that with Flemming material it gives a powerful and good stain, which is much more resistant to alcohol than that of the EHRLICH-BIONDI mixture, and is, therefore, much more adapted to ordinary work. The stain keeps well.

ISRAEL (*Praktik. Path. Hist.*, Berlin, 1893, p. 68) gives a more complicated receipt.

312. **Methyl Green and Eosin** (CALBERLA, *Morph. Jahrb.*, iii, 1877, Heft 3, p. 625; LIST, *Zeit. wiss. Mik.*, ii, 1885, p. 147; BALBIANI, *Ann. Microgr.*, Paris, vii, 1895, p. 245; RHUMBLER, *Zeit. wiss. Zool.*, lxi, 1895, p. 38).—See early editions.

313. **Methylen Blue and Eosin** (CHENZINSKY, quoted from *Zeit. wiss. Mik.*, xi, 2, 1894, p. 269).

Methylen blue, sol. sat. in water	40
Eosin, 0·5 per cent. in 70 per cent. alcohol	20
Distilled water, or glycerin	40

This solution will only keep for about eight days.

PIANESE (*ibid.*, xi, 1894, p. 345) adds a considerable proportion of carbonate of lithia.

See also the mixture of BREMER (*Arch. mik. Anat.*, xlv, 1895, p. 446).

I have tried CHENZINSKY'S mixture as a tissue stain, without good results; but see ROSIN, *Berliner klin. Wochenschr.*, 1898, p. 251; *Zeit. wiss. Mik.*, xvi, 1899, p. 223, and xvii, 1900, p. 333.

See also LAURENT (*Centralb. allg. Path.*, xi, 1900, p. 86; *Zeit. wiss. Mik.*, xvii, 1900, p. 201).

314. MALLORY'S **Eosin and Methylen Blue** (*Journ. med. Research*, January, 1904).—Sections of ZENKER material (other sublimate material not so good) are stained for half to three quarters of an hour at 56° C. in 5 per cent. aqueous solution of eosin, rinsed and flooded with solution

of one part of methylen blue, and one of potassium carbonate in 100 of water, diluted with about seven parts of water. After forty minutes they are flooded (not washed) with water, and differentiated for about 5 minutes in alcohol of 95 per cent. Absolute alcohol, xylol, balsam.

315. **Other Eosin and Methylen-blue Stains.**—For some very important ones see under "Blood."

316. **Light Green (Lichtgrün S. F.).**—An "acid" colour, soluble in water or alcohol, and a good plasma stain.

BENDA (*Verh. physiol. Ges. Berlin*, Dec. 18th, 1891, Nos. 4 u. 5) stains sections for twenty-four hours in anilin-water safranin solution, then for about half a minute in a solution of 0·5 grm. Lichtgrün or **Säureviolett** (Grübler) in 200 c.c. of alcohol, dehydrates and mounts in balsam. This process gives a very elegant stain, but requires very thin sections, and there is always risk of the safranin being washed out. The Lichtgrün stain unfortunately does not keep at all well.

See also PRENANT, *Arch. mik. Anat.*, vii, 1905, p. 430, and GUIEYSSE, *C.R. Soc. Biol.*, lxii, 1907, p. 1212.

317. **Janus Green** (MICHAELIS, *Arch. mik. Anat.*, lv, 1900, p. 565).—Used in solution of 1 : 30,000 for staining certain granules (pancreas, salivary glands, etc.) in the fresh state.

318. **Malachite Green (syn. Solid Green, Victoria Green, New Green, Benzoyl Green, Fast Green).**—A basic colour, which has been used as a plasma stain for the ova of *Ascaris* by VAN BENEDEN and NEYT. These authors used it for glycerin preparations; it can hardly be got into balsam.

FLEMMING (*Arch. mik. Anat.* xix, 1881, p. 324) attributes to it a special affinity for nucleoli.

319. **Iodine Green ("HOFMANN'S Grun")**, see GRIESBACH (*Zool. Anz.*, No. 117, vol. v, 1882, p. 406).—Stain essentially that of methyl green, but plasma often violet through the presence of a violet impurity (MAYER, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 311; see also earlier editions). It is now only used by botanists.

320. **Thiophen Green (Thiophengrün)**, see KRAUSE, *Intern. Monatsschr. Anat., etc.*, iv, 1887, Heft 2.

321. **Coerulein S.**, a green "acid" dye, is recommended for the staining of muscle-fibrils by M. v. LENHOSSÉK (*Anat. Anz.*, xvi, 1899, p. 339).—See also HEIDENHAIN, *ibid.*, xx, 1901, p. 37, and RAWITZ, *ibid.*, xxi, 1902, p. 554.

322. Quinoleïn Blue (Cyanin, Chinolinblau; v. RANVIER, *Traité*, p. 102).—Quinoleïn is said by Ranvier to have the property of staining fatty matters an intense blue.

It is useful for staining Infusoria, which in dilute solution it stains during life. See the methods of CERTES.

From the reactions mentioned by Ranvier it would seem that his "bleu de quinoléine" is not the preparation that usually goes under that name. See EHRLICH, in *Arch. mik. Anat.*, xiii, 1877, p. 266.

323. Indulin and Nigrosin.—**Indulin, Nigrosin, Indigen, Coupier's Blue, Fast Blue R, Fast Blue B, Blackley Blue, Guernsey Blue, Indigo substitute** are the names of brands of a group of dyes, mostly "acid," related to the base violanilin. According to BEHRENS the name Indulin is generally given to a bluish brand, and that of Nigrosin to a blacker one.

Nigrosin, used with sublimate material, I find stains both nuclei and cytoplasm, the chromatin strongly. It will not give the stain at all with chrom-osmium material.

According to CALBERLA (*Morph. Jahrb.*, iii, 1877, p. 627) the concentrated aqueous solution of *Indulin* should be diluted with six volumes of water. Sections will stain in the dilute solution in five to twenty minutes. He also says that it never stains nuclei; the remaining cell-contents and intercellular substance are stained blue. This seems to me to be, roughly, correct.

324. Safranin and Nigrosin (or Indigo-Carmine) (KOSSINSKI, *Zeit. wiss. Mik.*, vi, 1880, p. 61).—See early editions.

325. Picro-Nigrosin, PFITZER (*Deutsch. Botan. Gesellsch.*, 1883, p. 44) dissolves nigrosin in a saturated solution of picric acid in water, and uses it for fixing and staining at the same time, on the slide. See also under "Connective Tissues."

326. Anilin Blue.—Under this title are comprised various "basic" derivatives of the base rosanilin. They occur under the names **Spirit Soluble Blue (Bleu Alcool), Gentian Blue 6 B, Spirit Blue O, Opal Blue, Bleu de Nuit, Blue Lumière, Parma Bleu, Blue de Lyon.** Some authors give the name Bleu de Nuit and Gründstichblau as synonyms of **Bleu de Lyon.** The *Encycl. mik. Technik.* says it is "Anilinblau B— 6 B," with many synonyms, or designations of brands, Parma blue being "Anilinblau R or 2 R." I find it a fairly good stain, giving very good differentiations of nerve-tissue and of cartilage (as has already been pointed out by BAUMGARTEN and by JACOBY). MAURICE and SCHULGIN stain in bulk with it after borax-carmine, using a very dilute alcoholic solution.

BAUMGARTEN and JACOBY stain sections in a 0·2 per cent. alcoholic solution.

TONKOFF (*Arch. mik. Anat.*, lvi, 1900, p. 394) adds a little tincture of iodine to the solution of the dye, or mordants the sections with iodine.

SKROBANSKY (*Intern. Monatsschr. Anat.*, xxi, 1904, p. 20) uses it in water with picric acid.

327. **Carmine Blue** (**Bleu Carmin Aqueux**, from Meister, Lucius, and Brunig, at Höchst-a-M.).—JANSSENS (*La Cellule*, ix, 1893, p. 9) states that this colour possesses a special affinity for the parts of cytoplasm that are undergoing cuticular differentiation. He uses it in alcoholic solution acidified.

328. **Methyl Blue.**—Under this title are comprised some other derivatives of the base rosanilin. They are "acid" colours. Here belong **Methyl Blue, Cotton Blue, Water Blue** (**Wasserblau**), **Methyl Water-Blue, China Blue** (**Chinablau**), **Soluble Blue.**

Amongst these **Water Blue** (**Wasserblau**) possesses some useful properties. According to MITROPHANOW (quoted from *Zeit. wiss. Mik.*, v, 1888, p. 513), used in concentrated aqueous solution it gives a very good double stain with safranin. It is very resistant to alcohol. Using the Wasserblau first, and then the safranin, I have had some interesting results. The Wasserblau must be used first. With chrom-osmium material, twelve to twenty-four hours in the blue, and four or five in the safranin, may not be too much. My stains have not kept well.

MANN (*Methods*, etc., p. 216) uses a mixture of 35 parts 1 per cent. solution of eosin, 45 of methyl blue, and 100 of water. He has also (*Zeit. wiss. Mik.*, xi, 1894, p. 490) used a similar mixture for nerve-cells.

329. **Anilin Blue-black.**—A preparation cited under this name has been recommended by BEVAN LEWIS and others for nervous tissue. The dye used by them cannot now be identified. Dr. GRÜBLER writes me that the anilin blue-black of his list is the oxyazo colour blue-black B or AZOSCHWARZ; but that dye had not been discovered when Bevan Lewis wrote. See also HEIDENHAIN in *Zeit. wiss. Mik.*, xx, 1903, p. 185, and xxv, 1909, p. 407.

330. **Violet B** (or **Methyl Violet B**) (S. MAYER, *Sitzb. k. k. Akad. wiss. Wien*, iii Abth, February, 1882).—Used in solutions of 1 grm. of the colour to 300 grms. of 0·5 per cent.

salt solution, and with *fresh tissues* that have not been treated with any reagent whatever, this colour gives a stain so selective of the elements of the vascular system that favourable objects, such as serous membranes, appear as if injected. The preparations do not keep well; acetate of potash is the least unsatisfactory medium for mounting them in, or a mixture of equal parts of glycerine and saturated solution of picrate of ammonia (*Anat. Anz.*, 1892, p. 221). See also under "Plasmafibrils."

The allied dye, **Crystal Violet**, has been employed for staining sections, *e. g.* by KROMAYER and others. BENDA (*Neurol. Centralb.*, xix, 1900, p. 792) stains in a mixture of 1 vol. saturated sol. of the dye in 70 per cent. alcohol, 1 vol. 1 per cent. sol. of hydrochloric acid in 70 per cent. alcohol, and 2 vols. of anilin water, the liquid being warmed until vapour is given off, then cooled and the sections dried with blotting-paper, treated one minute with 30 per cent. acetic acid, dehydrated with alcohol and cleared with xylol.

331. **Kresyl Violet.**—An oxyazin dye, giving metachromatic stains. HERXHEIMER (*Arch. mik. Anat.*, liii, 1899, p. 519, and liv, p. 289) stains sections of skin with **Kresyl-echtviolett.** Nuclei blue, plasma reddish. Similarly FICK (*Centralb. allg. Path.*, xiii, 1902, p. 987; *Zeit. wiss. Mik.*, xx, 1903, p. 223), staining for three or four minutes in a concentrated aqueous solution, and differentiating in alcohol until the connective tissue has become colourless. Keratohyalin violet-red to salmon-coloured.

332. **Säureviolett,** see § 316.

333. **Benzoazurin** may be made to give either a diffuse or a nuclear stain, according to MARTIN (see *Zeit. wiss. Mik.*, vi, 1889, p. 193).

334. RAWITZ' **"Inversion" Plasma Stains.**—It has been discovered by RAWITZ that by means of appropriate mordants certain basic anilins, which by the usual methods of regressive staining are pure chromatin stains, may be made to afford a pure plasma stain, thus giving an "inversion" of the usual stain. The stain, in my opinion, is a vile one. For details see *fourth edition*, or RAWITZ (*Sitzb. Gesnaturf. Freunde, Berlin*, 1894, p. 174; *Zeit. wiss. Mik.*, xi, 1895, p. 503; and his *Leitfaden f. hist. Untersuchungen*, Jena, 1895, p. 76).

335. **Artificial Alizarin** (RAWITZ, *Anat. Anz.*, xi, 10, 1895, p. 295).—A double stain by means of artificial Alizarin, or Alizarin-cyanin, requiring the use of special mordants supplied by the colour manufacturers, and very complicated. See *fifth edition.*

Rawitz (*Zeit. wiss. Mik.*, 1909, pp. 393 and 395) also recommends a solution of 1 grm. of **Säure-Alizarinblau BB** (or **Säuregrün G**) (both from Höchst), 10 grms. ammonia alum, 100 c.c. glycerin, and 100 c.c. water.

Szütz (*ibid.*, xxix, 1912, p. 289) fixes in a mixture of 15 c.c. 1 per cent. platinum chloride, 15 c.c. formol, and 30 c.c. saturated solution of sublimate, makes paraffin sections, and stains them with Heidenhain's iron-hæmatoxylin. They are then treated for five to six hours with 5 per cent. solution of aluminium acetate, rinsed, and stained for five to six hours with Benda's sulphalizarinate of soda (given under "Mitochondria"), and got into balsam. A red plasma stain, affecting plasmafibrils. For *intra vitam* stains with alizarin see § 208 (Fischel), and Nilsson, *Zool. Anz.*, xxxv, 1909, p. 196.

336. For Benda's **Alizarin Stains**, see under "Centrosomes," "Mitochondria," and "Neuroglia."

CHAPTER XVI.

METHYLEN BLUE.

337. **Methylen Blue** is a "basic" dye, being the chloride or the zinc chloride double salt of tetramethylthionin. It appears that some persons have confounded it with the "acid" dye methyl blue, to which it has not, histologically, any resemblance.

Commercial methylen blue sometimes contains as an impurity a small quantity of a reddish dye, which used to be taken to be methylen red. This impurity is present from the beginnning in many brands of methylen blue,. is frequently developed in solutions of the dye that have been long kept (so-called "ripened" solutions), and is still more frequently found in kept *alkaline* solutions. According to NOCHT (*Centralb. Bakteriol.*, xxv, 1899, pp. 764–769; *Zeit. wiss. Mik.*, xvi, 1899, p. 225) it is not methylen red, nor methylen violet either, but a new colour, for which NOCHT proposes the name "Roth aus Methylenblau."

According to MICHÆLIS (*Centralb. Bakteriol.*, xxix, 1901, p. 763, and xxx, 1901, p. 626; *Zeit. wiss. Mik.*, xviii, 1902, p. 305, and xix, 1902, p. 68) confirmed later by NOCHT, REUTER, and GIEMSA, this dye is **Methylenazur**, an oxidation-product of methylen blue, already described by BERNTHSEN in 1885. It is an energetic dye, of markedly metachromatic action, and to it are due the metachromatic effects of methylen blue solutions (methylen blue itself is not metachromatic).

The presence of this dye as an impurity in methylen blue is not always an undesirable factor; on the contrary, it sometimes affords differentiations of elements of tissues or of cells that cannot be produced by any other means. Methylen blue that contains it is known as *polychrome methylen blue*, and is employed for staining *certain cell-granules*. UNNA (*Zeit. wiss. Mik.*, viii, 1892, p. 483) makes this as follows:

A solution of one part of methylen blue and one of carbonate of potash in twenty of alcohol and a hundred of water is evaporated down to a hundred parts. (It may be used at once, or after diluting with an equal volume of anilin water, for sections, which after staining may be differentiated with glycol, creosol, or Unna's glycerin-ether mixture—all of which, as well as the polychrome methylen blue, can be obtained from Grübler & Hollborn.) MICHAELIS (*op. cit.*) makes it as follows: 2 gr. of medicinal methylen blue are dissolved in 200 c.c. of water, and 10 c.c. of $\frac{1}{10}$ normal solution of caustic soda added. Boil for a quarter of an hour; after cooling add 10 c.c. of $\frac{1}{10}$ normal sulphuric acid, and filter.

Methylenazur is isolated from methylen blue by the prolonged action of an alkali or of silver oxide. It seems also that it is formed in certain mixtures of methylen blue with eosin (ROMANOWSKY, LAVERAN, GIEMSA and others), by means of the eosin, which in these mixtures acts *chemically*, and can be replaced by resorcin, hydroquinon, and the like. It is best procured from Grübler & Hollborn, who supply it pure as "Azur I," and mixed with an equal quantity of methylen blue as "Azur II." See further as to this dye under "Stains for Blood."

There are several sorts of methylen blue sold, the most important being—"methylen blue, according to EHRLICH"; "methylen blue, according to KOCH"; "methylen blue BX, according to S. MAYER"; "Methylenblau, medic. pur."

The colour to be employed for *intra-vitam nerve staining* should be *as pure as possible*. APÁTHY (*Zeit. wiss. Mik.*, ix, 1893, p. 466) writes that the best—in fact, the only one that will give *exactly* the results described by him—is that of E. MERCK, of Darmstadt, described as "*medicinisches* Methylenblau." DOGIEL (*Encycl. mik. Technik.*, 1st edition, p. 811) has had his best results with "Methylenblau n. Ehrlich," or "BX," obtained from Grübler & Hollborn.

338. **The Uses of Methylen Blue.**—As a histological reagent it is used for sections of hardened central nervous tissue, in which it gives a specific stain of medullated nerves. It gives more or less specific stains of the basophilous granulations of "Mastzellen" and plasma-cells, and the granules of NISSL in nerve-cells, also mucin. It is much used—in the form of mixtures affording methylen azur—in the study of

blood, blood parasites, and similar objects. For all of these see the respective sections in Part II. Further, it stains a large number of tissues *intra vitam*, with little or no interference with their vital functions. And last, not least, it can be made to furnish stains of nerve tissue, intercellular cement substances, lymph spaces, and the like, that are essentially identical with those furnished by a successful impregnation with gold or silver, and are obtained with greater ease and certainty; with this difference, however, that gold stains a larger number of the nervous elements that are present in a preparation, sometimes the totality of them; whilst methylen blue stains only a selection of them, so bringing them more prominently before the eye, and allowing them to be traced for greater distances. These two uses form the subject of this chapter.

339. **Staining *in toto* during Life.**—Small and permeable aquatic organisms may be stained during life by adding to the water in which they are confined enough methylen blue to give it a very light tint. After a time they will be found to be partially stained—that is, it will be found that certain tissues have taken up the colour, others remaining colourless. If now you put back the animals into the tinted water and wait, you will find after a further lapse of time that further groups of tissues have become stained. Thus it was found by Ehrlich (*Biol. Centralb.*, vi, 1886, p. 214; *Abh. k. Akad. Wiss. Berlin*, February 25th, 1885) that on injection of the colour into living animals axis-cylinders of *sensory* nerves stain, whilst *motor* nerves remain colourless. [The motor nerves, however, will also stain, though later than the sensory nerves.] It might be supposed that by continuing the staining for a sufficient time, a point would be arrived at at which *all* the tissues would be found to be stained. This, however, is not the case. It is always found that the stained tissues only keep the colour that they have taken up for a short time after they have attained the maximum degree of coloration of which they are susceptible, and then begin to discharge the colour even more quickly than they took it up. According to Ehrlich this decoloration is explained as follows: methylen blue, on contact with reducing agents in alkaline solution, can be reduced to a colourless

body, its "leucobase." Now living or recently dead tissue elements are, or may be, both alkaline and very greedy of oxygen, and thus act on the dye as reducing agents. The leucobase thus formed is easily reoxidised into methylen blue by oxidising substances, or acids, or even by the mere contact of air—which latter property is taken advantage of in practice.

It follows that a total stain of all the tissues of a living intact organism can hardly be obtained under these conditions, but that a specific stain of one group or another of elements may be obtained in one of two ways. If the tissue to be studied be one that stains earlier than the others, it may be studied during life at the period at which it alone has attained the desired intensity of coloration. If it be one that stains later than the others, it may be studied at the period at which the earlier stained elements have already passed their point of maximum coloration and have become sufficiently decoloured, the later stained ones being at a point of desired intensity. Or the observer may fix the stain in either of these stages and preserve it for leisurely study by means of one of the processes given § 343.

The proper strength of the very dilute solutions to be employed for the staining of living organisms must be made out by experiment for each object. I think the tint is practically a sufficient guide, but it may be stated that when in doubt a strength of 1 : 100,000 may be taken, and increased or diminished as occasion may seem to require. Zoja (*Rendic R. Ist. Lombardo*, xxv, 1892; *Zeit. wiss. Mik.*, ix, 1892, p. 208) finds that for Hydra the right strength is from 1 : 20,000 to 1 : 10,000.

The stain is capricious. It is not possible to predict without trial which tissues will stain first in any organism. The stain penetrates very badly, which is no doubt one cause of its capriciousness. Gland cells generally stain early; then, in no definable order, other epithelium cells, fat cells, plasma cells, "Mastzellen," blood and lymph corpuscles, elastic fibres, smooth muscle, striated muscle. There are other elements that stain in the living state, but *not* when the staining is performed by *simple immersion of intact animals* in a dilute staining solution in the manner we are considering. Chief amongst these are *nerve-fibres* and *ganglion-cells, which* *remain unstained in the intact organism.* To get these stained, it is necessary to isolate them sufficiently, as explained in the following sections.

340. Staining Nervous Tissue during Life.—It was made out by Ehrlich (*op. cit.*, last §) that by injecting a solution of methylen blue into the vessels or tissues of living animals and shortly afterwards cutting out and examining small pieces of their tissues, these will be found to be intensely stained in some of their elements (chiefly nervous). If the tissues are mounted under a cover-glass, the stain will fade in a short time; but if the cover-glass be removed, so that oxygen can have access to the tissues, the stain will be restored, as explained last §. The chief elements stained in this way are peripheral nerves, and amongst these more especially axis-cylinders of sensory nerves.

Ehrlich held that the stain so obtained is a product of a *vital* reaction of the tissues, and that it cannot be obtained with dead material. Dogiel, however (*Arch. mik. Anat.*, xxxv, 1890, pp. 305 *et seq.*), found that muscle nerves of limbs of the frog could be stained as much as from three to eight days after the limbs had been removed from the animal. He concludes, indeed, that the reaction shows that the nerves were still living at that time. But it seems more natural to conclude with Apáthy (*Zeit. wiss. Mik.*, ix, 1892, pp. 15 *et seq.*) that nerve-tissue can be stained after life has ceased. Apáthy has directly experimented on this point, and sums up the necessary conditions as follows: The tissue need not be living, but must be fresh; nothing must have been extracted from it chemically, and its natural state must not have been essentially changed by physical means. For example, the tissue must not have been treated with even dilute glycerin, nor with alcohol, though a treatment for a short time with physiological salt solution is not very hurtful; it must not have been coagulated by heat. Michailow (*ibid.*, xxvii, 1910, p. 7) prefers tissues that have lain from one and a half to two hours after the death of the subject in Ringer's salt solution.

As above explained, the primary stain obtained by injecting methylen blue, or immersing tissues in it, only lasts a very short time. In order to get it to last long enough for study, it must be re-blued by oxidation (see last §). It is therefore the usual practice to dissect out the tissues to be examined, and leave them for some time exposed to the air. This is done in order that they may take up from the air the necessary *oxygen*. Another consideration that justifies the practice is that by exposure to air the preparations take up a trace of *ammonia*, and Apáthy has experimentally established that this is an important factor in the sharpness of the stain. Ehrlich also (*op. cit.*) holds that an alkaline reaction of the tissues is a necessary condition to the stain. Apáthy further holds that the stain is a *regressive* one, easily washed out by the surrounding liquid; and in order to prevent this washing-out being excessively rapid, it is desirable to have it go on in presence of as little liquid as possible.

341. The Modes of Staining.—The practice of the earlier workers at this subject was (following Ehrlich) to *inject* methylen blue into the vascular system or body-cavity of a living animal, wait a sufficient time, then remove the organ for further preparation and study. And there appears to have been a belief with some workers that it was essential that the stain should have been brought about by injection of the colouring matter into the *entire animal*. It is now known that the reaction can often be equally well obtained by removing an organ and subjecting it to a *bath* of the colouring matter in the usual way. But in some cases it seems that injection is preferable, if not necessary.

342. The Solutions employed.—The solutions used *for injection* are generally made in salt solution (physiological, or a little weaker); those for staining by immersion, either in salt solution or other "indifferent" liquid, or in pure water. The earlier workers generally took concentrated solutions. Thus Arnstein (*Anat. Anz.*, 1887, p. 125) injected 1 c.c. of saturated (*i. e.* about 4 per cent.) solution into the vena cutanea magna of frogs, and removed the organ to be investigated after the lapse of an hour. Biedermann (*Sitzb. Akad. Wiss. Wien, Math. Nat. Cl.*, 1888, p. 8) injected 0·5 to 1 c.c. of a nearly saturated solution in 0·6 per cent. salt solution into the thorax of crayfishes, and left the animals for from two to four hours before killing them. S. Mayer (*Zeit. wiss. Mik.*, vi, 1889, p. 423) took a strength of 1 : 300 or 400 of 0·5 per cent. salt solution. The solutions of Retzius are of the same strength. But the tendency of more recent practice is decidedly towards the employment of weaker solutions. Apáthy (*ibid.*, ix, 1892, pp. 25, 26 *et seq.*) finds that it is not only superfluous, but positively disadvantageous, to take solutions stronger than 1 : 1000. Dogiel (*Encycl. Mik. Technik.*, 1st ed., p. 815) recommends $\frac{1}{8}$ to $\frac{1}{4}$ per cent., or at most $\frac{1}{2}$ per cent. For warm-blooded animals the solution should be warmed to 36° or 37° C., and before sending in the injection the blood-vessels should be well washed out with similarly warmed salt solution. The injected organs may be removed after 20 to 30 minutes. They should be placed on a thin layer of spun glass moistened with weak ($\frac{1}{8}$ to $\frac{1}{15}$ per cent.) methylen blue, or simply spread out on a slide, and the whole placed in a Petri dish with a layer of the methylen blue on the bottom. The dish is best placed in a stove at 36° C., and after 15 to 30 minutes (if the pieces are thin) or 1 hour to $1\frac{1}{2}$ hours (if they are thick) specimens may be removed for examination or preservation ; or, without using the stove, specimens may be removed 10 to 20 minutes after injection, placed on a slide, and moistened with weak methylen blue or salt solution, and brought under the microscope. Then as soon as the stain is sufficiently brought out (40 to 60 minutes) they may be fixed (§ 343).

For staining *by immersion* the solutions should, if anything, be still weaker. Dogiel (*Arch. mik. Anat.*, xxxv, 1890, p. 305) places objects in a few drops of aqueous or vitreous humour, to which are added two or three drops of a $\frac{1}{16}$ to $\frac{1}{15}$ per cent. solution of methylen blue in physiological (0·75 per cent.) salt solution, and exposes them therein to the air. In thin pieces of tissues the stain begins to take effect in five or ten minutes, and attains its maximum in from fifteen to twenty minutes. For thicker specimens—retina, for instance—several hours may be necessary. The reaction is quickened by putting the preparations into a stove kept at 30° to 35° C. Rouget (*Compt. Rend.*, 1893, p. 802) employed a 0·05 per cent. solution in 0·6 per cent. solution (for muscles of Batrachia). Allen (*Quart. Journ. Micr. Sci.*, 1894, pp. 461, 483) takes for embryos of the lobster a solution of 0·1 per cent. in

0·75 per cent. salt solution, and dilutes it with 15 to 20 volumes of sea-water. Seidenmann (*Zeit. wiss. Mik.*, xvii, 1900, p. 239) takes for the choroid a solution of 0·02 per cent. in 0·5 per cent. salt solution. Lavdowsky (*ibid.*, xii, 1895, p. 177) takes $\frac{1}{10}$ to $\frac{1}{4}$ per cent. in white of egg, or serum. Similarly Young (*ibid.*, xv, 1898, p. 253). Michailow (*ibid.*, xxvii, 1910, p. 10) takes $\frac{1}{8}$ to $\frac{1}{32}$ per cent. in Ringer's salt solution (for nerves of Mammals).

Apáthy (*Zeit. wiss. Mik.*, ix, 1892, p. 15; see also his *Mikrotechnik*, p. 172) proceeds as follows for *Hirudinea* and other invertebrates. A portion of the ventral cord is exposed, or dissected out. If it be desired to stain as many ganglion cells as possible, as well as fibres, the lateral nerves, as well as the connectives, should be cut through near a ganglion. The preparation is then treated with the stain. This is, for the demonstration chiefly of fibres in *Hirudo* and *Pontobdella*, either a 1 : 1000 solution in 0·5 to 0·75 per cent. salt solution, allowed to act for ten minutes; or a 1 : 10,000 solution allowed to act for an hour to an hour and a half; or a 1 : 100,000 solution allowed to act for three hours (*Lumbricus* requires twice these times; *Astacus* and *Unio* require three times; medullated nerves of vertebrates four times). For the demonstration of ganglion cells the stain is allowed to act three or four times as long.

The preparations from the 1 : 1000 solution are then washed in salt solution for an hour; those from the 1 : 10,000 solution for a quarter of an hour; those from the 1 : 100,000 solution need not be washed at all. They are then treated with one of the ammoniacal fixing and differentiating liquids described in § 343. This is done by pouring the liquid over them, and leaving them in it *without moving them about in it* for at least an hour, and by preference in the dark. The further treatment is as described in § 343.

The object of the ammonia in these liquids is to *differentiate* the stain—to produce an artificial "secondary differentiation." It acts by washing out the absorbed colour from certain elements, others resisting longer.

See also, for Hirudinea, Sánchez, in *Trab. Lab. Invest. Biol. Univ. Madrid*, vii, 1909, fasc. 1–4, or *Zeit. wiss. Mik.*, xxvii, 1910, p. 393 (injection of solutions of 0·2, 0·1, or 0·05 per cent., with further treatment as Apáthy or Bethe).

343. **Fixation of the Stain.**—The stain obtained by any of these methods may be fixed, and more or less permanent preparations be made by one or other of the following methods :

Arnstein (*Anat. Anz.*, 1887, p. 551) puts the tissue for half an hour into saturated aqueous solution of picrate of ammonia.

S. Mayer (*Zeit. wiss. Mik.*, vi, 1889, p. 422) preferred a mixture of equal parts of glycerine and saturated picrate of ammonia solution, which served to fix the colour and mount the preparations in. This was also in principle the method of Retzius (*Intern. Monatschr. Anat. Phys.*, vii, 1890, p. 328).

Dogiel (*Encycl. mik. Techn.*, ii, p. 105) puts for 2 to 24 hours into saturated aqueous picrate of ammonia, and then into equal parts of glycerin and the picrate solution. (Thin membranes, and the like, may be fixed with 1 or 2 per cent. of 2 per cent. osmic acid solution added to the picrate solution and stained with picro-carmine before putting into the glycerin mixture.)

Other workers have employed saturated solution of iodine in iodide of potassium (so Arnstein) or picro-carmine (so Feist, *Arch. Anat. Entwickel.*, 1890, p. 116 ; cf. *Zeit. wiss. Mik.*, vii, 1890, p. 231), the latter having the advantage of preserving the true blue of the stain if it be not allowed to act too long, and the preparation be mounted in pure glycerin.

Picric acid has been used by Lavdowsky, but this after careful study is rejected by Dogiel.

Apáthy (*op. cit.*, § 342) brings preparations either into a concentrated aqueous solution of picrate of ammonia *free from picric acid*, and containing five drops of concentrated *ammonia* for every 100 c.c. ; or, which is generally preferable, into a 1 to 2 per cent. freshly prepared solution of neutral *carbonate of ammonia* saturated with picrate. They remain in either of these solutions, preferably in the dark, for *at least an hour*. They are then brought into a *small* quantity of saturated solution of picrate of ammonia in 50 per cent. glycerin, where they remain until thoroughly saturated. They are then removed into a saturated solution of the picrate in a mixture of 2 parts 50 per cent. glycerin, 1 part cold saturated sugar solution, and 1 part similarly prepared gum-arabic solution. When thoroughly penetrated with this they are removed and mounted in the following gum-syrup medium (*loc. cit.*, p. 37):

Picked gum-arabic . . .	50 grms.
Cane-sugar (not candied) . .	50 ,,
Distilled water	50 ,,

Dissolve over a water-bath and add 0·05 grm. thymol. (This mounting medium sets quickly and as hard as balsam, so that no cementing of the mounts is necessary. Farrants' medium [with omission of the arsenious acid] will also do. In neither case should either ammonium picrate or methylen blue be added to the medium.) Preparations that have been *fully* differentiated (§ 342) do not keep more than a few weeks; whilst those in which the differentiation has not been carried to the point of thorough tinctorial isolation of the neuro-fibrils have kept for five or six years (Apáthy, *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 712).

Pleschko (*Anat. Anz.*, xiii, 1897, p. 16) fixes with picrate, and then puts into 10 per cent. formol for a few days.

The methods described next § are also available for material not destined to be sectioned.

344. Methods for Sections.—The preceding methods do not give preparations that will resist the operations necessary for imbedding in paraffin or mounting in balsam. A strong solution of platinum chloride is said to do this (see Feist, *Arch. Anat. Entw.*, 1890, p. 116), but the preparations are not very satisfactory.

For the earlier method of Parker (*Zool. Anzeig.*, 1892, p. 375) with methylal see early editions. Later (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 4) he fixes the stain by dehydrating the objects in successive alcohols of 30, 50, 70, 95, and 100 per cent. strength, each containing 8 per cent. of corrosive sublimate, then brings them into a mixture of the last with an equal volume of xylol, and lastly into pure xylol.

For the earlier method of Bethe (*Arch. mik. Anat.*, xliv, 1894, p. 585), see last edition.

Bethe's later method (*Anat. Anz.*, xii, 1896, p. 438) is as follows: After staining, pieces of tissue of 2 to 3 mm.

thickness are treated for ten to fifteen minutes with a concentrated aqueous solution of picrate of ammonia and then brought into a solution of 1 grm. of molybdate of ammonium, either in 20 of water, or in 10 of water and 10 of 0·5 per cent. osmic acid or 2 per cent. chromic acid; or into a solution of phosphomolybdate of sodium in the same proportions, each of these solutions having added to it 1 drop of hydrochloric acid, and if desired 1 grm. of peroxide of hydrogen. They remain in one of these solutions for three quarters to one hour (or from four hours to twelve in the osmic acid one), and are then passed through water, alcohol, xylol, balsam, or paraffin. (The objects that have been treated with one of the solutions of the sodium salt are not thoroughly resistant to alcohol, so that for them it is well to cool the alcohol to under 15° C.) Sections may be after-stained with alum carmine, or "neutral" tar colours.

Slight modifications of this method are given by DOGIEL (*Arch. mik. Anat.*, xlix, 1897, p. 772; liii, 1898, p. 237; *Zeit. wiss. Zool.*, lxvi, 1899, p. 361; and *Encycl. mik. Technik*, 1903, p. 825, and 1910, p. 108). He omits the peroxide, the hydrochloric acid, and the cooling. Bethe (*Zeit. wiss. Mik.*, xvii, 1900, p. 21) does not approve of these modifications.

Further modifications of the molybdenum method have been published by LEONTOWITSCH (*Intern. Monatsschr. Anat.*, xviii, 1901, p. 142).

MICHAILOW (*Zeit. wiss. Mik.*, xxvii, 1910, p. 19) adds to 8 per cent. solution of molybdate 0·5 per cent. of formalin, leaves the objects in a large quantity of it (filtered) for 24 hours at 37° C., washes with warm water, and passes through alcohol and xylol into xylol-damar (not balsam).

See also SCHMIDT (*Arch. Ges. Phys.*, ciii, 1906, p. 522).

HARRIS (*Philadelphia Medical Journ.*, May 14th, 1898), after staining, rinses with water, and brings into a saturated solution of either ferrocyanide or ferricyanide of potassium which has been cooled to within a few degrees of zero (a trace of osmic acid may be added to prevent maceration). They remain therein for three to twenty-four hours, and are then washed in distilled water for an hour, and are dehydrated in absolute alcohol kept at a low temperature, cleared in xylol or cedar oil, and imbedded in paraffin.

345. Impregnation of Epithelia, Lymph-spaces, etc. (Dogiel, *Arch. mik. Anat.*, xxxiii, 1889, p. 440 *et seq.*).—Suitable pieces of tissue (thin membrane by preference) are brought fresh into a 4 per cent. solution of methylen blue in physiological salt solution (in the *Encycl. mik. Technik*, 1903, p. 827, Dogiel gives the strength of the methylen blue as ½ to 1 per cent.). After a few minutes therein they are brought into saturated solution of picrate of ammonia, soaked therein for half an hour or more, then washed in fresh picrate of ammonia solution, and examined in dilute glycerin.

If it be wished only to demonstrate the outlines of endothelium cells, the bath in the stain should be a short one, not longer than ten minutes in general; whilst if it be desired to obtain an impregnation of ground-substance of tissue, so as to have a negative image of juice canals or other spaces, the staining should be prolonged to fifteen or thirty minutes.

If it be desired to preserve the preparations permanently, they had better be mounted in glycerin saturated with picrate of ammonia, or (*Encycl.*, 1910, ii, p. 110) fixed with ammonium molybdate and a trace of osmium.

The effect is practically identical (except as regards the colour) with that of a *negative impregnation with silver nitrate.*

S. Mayer (*Zeit. wiss. Mik.*, vi, 1889, p. 422) stains tissues for about ten minutes in a 1 : 300 or 400 solution of methylen blue in 0·5 per cent. salt solution, rinses in salt solution, and puts up in the glycerin-picrate of ammonia mixture given § 343. The images are generally positive after injection of the colour into the vascular system; negative after immersion of the tissues.

Timofejew (*Anat. Anz.*, xxxv, 1909, p. 296) impregnates for 15 to 20 minutes in a solution of 1 : 3000 or 4000 strength, fixes with a very weak solution of ammonium picrate in salt solution, and puts up in a mixture of 50 c.c. glycerin, 50 c.c. water, and 35 c.c. saturated solution of the picrate: or fixes with ammonium molybdate of 8 per cent. and mounts in balsam.

346. Toluidin Blue or Thionin as succedanea of methylen blue.—Harris (*Philadelphia Med. Journ.*, May 14th, 1898) has found that there is no reaction of methylen blue that cannot be equally well obtained with toluidin blue or thionin. For staining pieces of tissue he takes:

Toluidin blue, 0·1 per cent. sol. in physiological salt solution	2 parts
Ammonium chloride 0·25 per cent. in water	1 part
Egg albumen	1 ,,

For injections he uses 1 part of the dye to 1000 of physiological salt solution.

Any of the methylen blue fixing methods may be employed and the whole technique is the same.

L. Martinotti (*Zeit. wiss. Mik.*, xxvii, 1910, p. 24) recommends a *polychrome* toluidin blue, made by adding 0·5 per cent. of lithium carbonate to a 1 per cent. solution of the dye and keeping till a purple-red tone appears. Or, a stock solution made of 1 grm. toluidin blue, 0·5 grm. lithium carbonate, glycerin 20 grms., alcohol 5 grms., and water 75.

CHAPTER XVII.

METALLIC STAINS (IMPREGNATION METHODS).

347. The Characters of Impregnation Stains.—By impregnation is understood a mode of coloration in which a colouring matter is deposited in tissues in the form of a *precipitate*—the impregnated elements becoming in consequence opaque. By staining, on the other hand, is understood a mode of coloration in which the colouring matter is retained by the tissues as if in a state of *solution*, showing no visible solid particles under the microscope, the stained elements remaining in consequence transparent. But it is not right to draw a hard and fast line between the two kinds of coloration. Some of the metallic salts treated of in this chapter give, besides an impregnation, in some cases a true stain. And some of the dyes that have been treated of in the preceding chapters give, besides a stain, a true impregnation. Methylen blue, for instance, will give in one and the same preparation an impregnation and a stain; and in most gold chloride preparations the coloration is in places of the nature of a finely divided solid deposit, in others a perfectly transparent stain.

348. Negative and Positive Impregnations.—In a *negative* impregnation intercellular substances alone are coloured, the cells themselves remaining colourless or very lightly tinted. In a *positive* impregnation the cells are stained and the intercellular spaces are unstained. (A directly contrary statement, made in a recent *Lehrbuch*, is erroneous.)

Negative impregnation is generally held to be *primary* because brought about by the direct reduction of a metal in the intercellular spaces; positive impregnation to be *secondary* (in the case of silver nitrate at least) because it is brought about by the solution in the liquids of the tissues of the metallic deposit formed by a primary impregnation. and the consequent staining of the cells by the new solution of metallic salt thus formed. These secondary impregnations takes place when the reduction of the metal in the primary impregnation is not sufficiently energetic (see on these points HIS, *Schweizer Zeit. Heilk.*, ii, Heft 1, p. 1; GIERKE, *Zeit. wiss. Mik.*, i, p. 393; RANVIER, *Traité*, p. 107).

As to the nature of the black or brown deposit or stain formed in the intercellular spaces in cases of primary impregnation see SCHWALBE, *Arch. mik. Anat.*, vi, 1870, p. 5; GIERKE'S *Färberei zu mikroskopischen Zwecken*, in vols. i and ii of *Zeit. wiss. Mik.*; JOSEPH, *Sitzb. Akad. Wiss. Berlin*, 1888; *Zeit. wiss. Mik.*, xi, 1, 1894, p. 42 *et seq.* It evidently cannot consist of metallic silver, as it is soluble in hyposulphite of soda. See also MACALLUM, *Proc. Roy. Soc.*, lxxvi, 1905, p. 217, and ACHARD and REYNAUD, *C. R. Soc. Biol.*, lxi, 1906, p. 43

349. Action of Light on Solutions of Metallic Salts.—Stock solutions of metallic salts are generally kept in the dark, or at least in coloured bottles, under the belief that exposure to light reduces them. It has been pointed out in § 35 that in the case of osmic acid, not light, but *dust* is the reducing agent, and that solutions may be exposed to light with impunity if dust be absolutely denied access to them. I have now good evidence to the effect that the same is the case with other metallic solutions; and the point is raised whether such solutions are not positively improved for impregnation purposes by exposure to light! Dr. LINDSAY JOHNSON writes me as follows:

"One may (I find by experiment) state as a rule without exception that all the solutions of the chlorides and nitrates of the metals will keep indefinitely in clean white stoppered bottles in the sunlight; and as far as osmium, uranium, gold and silver, and platinum are concerned, actually improve or ripen by a good sunning. All photographers tell me their papers salt more evenly by old well-sunned silver nitrate than by a fresh solution kept in the dark; and I go so far as to say that this is one of the reasons why gold stains are so unsatisfactory."

APÁTHY (*Mitt. Zool. Stat. Neapel*, xii, 1897, p. 722) leaves his gold solutions exposed to light, so long as there are no tissues in them.

350. State of the Tissues to be Impregnated.—The majority of stains given by *dyes* are only obtained with tissues that have been changed in their composition by the action of fixing and preservative reagents. With metallic impregnations the case is different; perfectly *fresh* tissues—that is, such as are either living, or at all events have not been treated by any reagent whatever—will also impregnate with the greatest ease and precision. Indeed, some impregnations will not succeed at all with tissues that are not fresh in the sense above explained.

Silver.

351. **Silver Nitrate: Generalities.**—The principles of its employment are given by Ranvier (*Traité*, p. 105) as follows:

Silver nitrate may be employed either in solution or in the solid state. The latter method is useful for the study of the cornea and of fibrous tissues, but is not suitable for epithelia. For the cornea, for instance, proceed as follows: The eye having been removed, a piece of silver nitrate is quickly rubbed over the anterior surface of the cornea, which is then detached and placed in distilled water; it is then brushed with a camel's hair brush in order to remove the epithelium. The cornea is then exposed to the action of light. It will be found that the nitrate has traversed the epithelium and soaked into the fibrous tissue, on the surface of which it is reduced by the light. The cells of the tissues will be found unstained.

It is generally employed in solution, in the following manner: In the case of a membrane, such as the epiploön, the membrane must be *stretched* like a drum-head over a porcelain dish,* and *washed* first with distilled water, and then washed with a solution of silver nitrate. In order to obtain a powerful stain it is necessary that this part of the operation be performed in direct sunlight, or at least in a very brilliant light. As soon as the tissue has begun to turn of a blackish grey the membrane is removed, washed in distilled water, and *mounted* on a slide in some suitable examination medium.

---

* The **Hoggans Histological Rings** will be found much more convenient. They are vulcanite rings made in pairs, in which one ring just fits into the other, so as to clip and stretch pieces of membrane between them. They will be found described and figured in *Journ. Roy. Mic. Soc.*, ii, 1879, p. 357, and in Robin's *Journ. de l'Anat.*, 1879, p. 54. They may be obtained of Burge & Warren, 42, Kirby Street, Hatton Garden, London, E.C.

If the membrane were left in the water the cells would become detached, and would not be found in the finished preparation.

If the membrane had not been stretched as directed the silver would be precipitated not only in the intercellular spaces, but in all the small folds of the surface.

If the membrane had not been washed with distilled water before impregnation there would have been formed a deposit of silver on every spot on which a portion of an albuminate was present, and these deposits might easily be mistaken for a normal structure of the tissue. It is thus that impurities in the specimen have been described as stomata of the tissue.

If the solution be taken too weak—for instance, 1 : 500 or 1 : 1000, or if the light be not brilliant—a *general* instead of an *interstitial* stain will result; nuclei will be most stained, then protoplasm, and the intercellular substance will contain but very little silver. In general in a good "impregnation" the contents of the cells, and especially nuclei, are quite invisible.

The tissues should be constantly *agitated* in the silver-bath in order to avoid the formation on their surfaces of deposits of chlorides and albuminates of silver.

These impregnations only succeed with *fresh* tissues.

352. Silver Nitrate: the Solutions to be employed (RANVIER).—The solutions generally employed by RANVIER vary in strength from 1 : 300 to 1 : 500. Thus 1 : 300 is used for the epiploön, pulmonary endothelium, cartilage, tendon; whilst a strength of 1 : 500 is employed for the phrenic centre, and the epithelium of the intestine. For the endothelium of blood-vessels (by injection) solutions of 1 : 500 to 1 : 800 are taken.

M. DUVAL (*Précis*, p. 229) takes solutions of 1, 2, or at most 3 per cent.

v. RECKLINGHAUSEN used, for the cornea, a strength of from 1 : 400 to 1 : 500 (*Die Lymphgefässe*, etc., Berlin, 1862, p. 5).

ROBINSKI (*Arch. de Physiol.*, 1869, p. 451) used solutions varying between 0·1 and 0·2 per cent., which he allowed to act for thirty seconds.

Rouget (*Arch. de Physiol.*, 1873, p. 603) employed solutions as weak as 1 : 750, or even 1 : 1000, exposing the tissues to their action several times over, and washing them with water after each bath.

The Hertwigs take, for marine animals, a 1 per cent. solution (*Jen. Zeit. Naturk.*, xvi, pp. 313 and 324).

The Hoggans (*Journ. of Anat. and Physiol.*, xv, 1881, p. 477) take for lymphatics a 1 per cent. solution.

Tourneux and Herrmann (Robin's *Journal de l'Anat.*, 1876, p. 200) took for the epithelia of Invertebrates 3 : 1000, and in some cases weaker solutions,—for one hour, washing out with alcohol of 90 per cent.

Hoyer (*Arch. mik. Anat.*, 1876, p. 649) takes a solution of nitrate of silver, and adds ammonia to it until the precipitate that is formed just redissolves, then dilutes the solution until it contains from 0·75 to 0·50 per cent. of the salt. This *ammonio-nitrate* solution has the advantage of impregnating absolutely nothing but endothelium or epithelium ; connective tissue is not affected by it.

Ranvier's *injection-mass* for impregnating endothelium is given under "Injection."

Dekhuysen (*Anat. Anz.*, iv, 1889, No. 25, p. 789) has applied to terrestrial animals the method of Harmer for marine animals (§ 356). For details see *previous editions.*

Regaud (*Journ. Anat. et Phys.*, xxx, 1894, p. 719) recommends for the study of lymphatics a process devised by Renaut, for the details of which see also *previous editions.*

353. Other Salts of Silver.—Alferow (*Arch. Phys.*, i, 1874, p. 694) employs the picrate, lactate, acetate, and citrate, in solution of 1 : 800, and adds a small quantity of the acid of the salt taken (10 to 15 drops of a concentrated solution of the acid to 800 c.c. of the solution of the salt). This decomposes the precipitates formed by the action of the silver salt on the chlorides, carbonates, and other substances existing in the tissues.

Regaud and Dubreuil (*C.R. Ass. Anat.*, 5 Sess. 1903, p. 122) take a fresh solution of protargol or a mixture of equal parts of 1 per cent. protargol and 1 per cent. osmic acid, thus avoiding precipitates.

354. Silver Nitrate: Reduction.—Reduction may be effected in other media than distilled water.

v. Recklinghausen washed his preparations in salt solution before exposing them to the light in distilled water (*Arch.*

path. Anat., xix, p. 451). Physiological salt solution (0·75 per cent.) is commonly used for these washings.

Müller (*Arch. f. path. Anat.*, xxxi, p. 110), after impregnation by immersion for two or three minutes in a 1 per cent. solution of nitrate of silver in the dark, adds to the solution a small quantity of 1 per cent. solution of iodide of silver (dissolved by the aid of a little iodide of potassium). After being agitated in this mixture the preparations are washed with distilled water, and exposed to the light for two days in a 1 per cent. solution of nitrate of silver (see also Gierke, in *Zeit. wiss. Mik.*, i, 1884, p. 396).

Rouget (*Arch. de Physiol.*, 1873, p. 603) reduces in glycerin; Szütz (*Zeit. wiss. Mik.*, xxix, 1912, p. 291) in glycerin with $\frac{1}{10}$ of formol.

Sattler (*Arch. Mik. Anat.*, xxi, p. 672) exposes to the light for a few minutes in water acidulated with acetic or formic acid. Thanhoffer (*Das Mikroskop*, 1880) employs a 2 per cent. solution of acetic acid.

Krauss brings his preparations, after washing, into a light red solution of permanganate of potash. Reduction takes place very quickly, even in the dark.

Oppitz puts for two or three minutes into a 0·25 or 0·50 per cent. solution of chloride of tin.

Jakimovitch (*Journ. de l'Anat.*, xxiii, 1888, p. 142) brings nerve preparations, as soon as they have become of a dark brown colour, into a mixture of formic acid 1 part, amyl alcohol 1 part, and water 100 parts, and exposes to the light for five to seven days, the mixture being renewed from time to time.

Dekhuysen (*op. cit.*, last §) reduces in oil of cloves, after dehydration.

355. Fixation.—Legros (*Journ. de l'Anat.*, 1868, p. 275) washes his preparations, after reduction, in hyposulphite of soda, to prevent after-blackening. According to Duval (*Précis*, p. 230) they should be washed for a few seconds only in 2 per cent. solution and then in distilled water.

Gerota (*Arch. Anat. Phys., Phys. Abth.*, 1897, p. 428) reduces in a hydroquinone developing solution, followed by fixation in hyposulphite of soda, just as in photography.

356. Impregnation of Marine Animals.—On account of the chlorides that bathe the tissues of marine animals, these cannot be treated *directly* with nitrate of silver.

Hertwig (*Jen. Zeit.*, xiv, 1880, p. 322) recommends fixing them with a weak solution of osmic acid, then washing with distilled water until the wash-water gives no more than an insignificant precipitate with silver nitrate, and then treating for six minutes with 1 per cent. solution of silver nitrate.

Harmer (*Mitth. Zool. Stat. Neapel*, v, 1884, p. 445) washes them for some time (half an hour) in a 5 per cent. solution of nitrate of potash in distilled water; they may then be treated with silver nitrate in the usual way. For some animals he recommends a 4·5 per cent. solution of sulphate of soda.

357. Double-staining Silver-stained Tissues.—The nuclei of tissues impregnated with silver may be stained with the usual reagents, provided that solutions containing free ammonia be avoided. These stains will only succeed, however, with successful negative impregnations, as nuclei that have been impregnated will not take the second stain.

Impregnation with silver may be followed by impregnation with gold. In this case the gold generally substitutes itself for the silver in the tissues, and though the results are sharp and precise, the effect of a double stain is not produced. See hereon Gerota, *loc. cit.*, § 355.

358. Impregnation of Nerve Tissue.—*For this subject, which includes the important bichromate-and-silver method of* Golgi, *and the neurofibril methods of* Bielschowsky *and* Ramón y Cajal, *see Part II. These give important results, not only with Nervous tissue, but with various forms of Connective tissue, mitochondrial formations, etc.*

Gold.

359. The Characters of Gold Impregnations.—Gold chloride differs from nitrate of silver in that it generally gives *positive* (§ 348) impregnations only. It generally gives negative images only with such tissues as have first received a negative impregnation with silver, the gold substituting itself for the silver. In order to obtain these images you first impregnate very lightly with silver; reduce; treat for a few minutes with a 0·5 per cent. solution of gold chloride, and reduce in acidulated distilled water.

This process, however, is in but little use, and except for certain special studies on the cornea and on connective tissue, the almost exclusive function of gold chloride is the impregnation of nervous tissue, for which it exhibits a remarkable selectivity.

360. Pre-impregnation and Post-impregnation.—Gold methods may be divided into two groups: viz. *pre-impregnation* methods, characterised by employing *perfectly fresh* tissues, and *post-impregnation* methods, characterised by the employment of *fixed and hardened* tissues. Both are chiefly used for *nervous* tissue. They give in some respects opposite results. Pre-impregnation gives nuclei unstained, cytoplasm rather strongly stained, axis-cylinders reddish-violet. Post-impregnation gives nuclei sharply stained, cytoplasm pale, axis-cylinders black, and (when successful) showing their neurofibrils sharply distinguished from the interfibrillar substance.

In Apáthy's view (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 718) successful gold preparations should show a true *stain*, not an impregnation (§ 347), the stain being brought about by the formation of gold oxide (AuO) which combines with the tissue elements. He advises in consequence that preparations should *not be moved about* more than can be helped in the reducing bath, so that the colouring oxide may not be washed away from the tissues before the stain has taken effect.

361. As to the Commercial Salts of Gold.—Squire's *Methods and Formulæ*, etc. (p. 43), says: "Commercial chloride of gold is not the pure chloride, $AuCl_3$, but the crystallised double chloride of gold and sodium, containing 50 per cent. of metallic gold.

"Commercial chloride of gold and sodium is the above crystallised double chloride mixed with an equal weight of chloride of sodium, and contains 25 per cent. of metallic gold."

This, however, appears not to be the case in Germany.

Dr. GRÜBLER, writing to MAYER (see the *Grundzüge*, LEE und MAYER, p. 215), says: "*Aurum chloratum fuscum* contains about 53 per cent. Au, the *flavum* about 48 per cent.; in both of them there should be only water and hydrochloric acid besides the gold, no sodium chloride. Pure *Auronatrium chloratum* contains 14·7 per cent. of sodium chloride, though samples are found in commerce with much more."

APÁTHY (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 722) formerly employed the *aurum chloratum flavum*, but now prefers the *fuscum*.

A. *Pre-impregnation.*

362. The State of the Tissues to be impregnated.—The once classical rule, that for researches on nerve-endings the tissues should be taken perfectly fresh, seems not to be valid for all cases. For DRASCH (*Sitzb. Akad. Wiss. Wien*, 1881, p. 171, and 1884, p. 516; and *Abhand. math.-phys. Cl. K. Sach. Ges. Wiss.*, xiv, No. 5, 1887; *Zeit. wiss. Mik.*, iv, 1887, p. 492) finds that better results are obtained with tissues that have been allowed to lie after death for twelve, twenty-four, or even forty-eight hours in a cool place.

363. COHNHEIM'S Method (*Virchow's Arch.*, Bd. xxxviii, pp. 346—349; *Stricker's Handb.*, p. 1100).—Fresh pieces of cornea (or other tissue) are put into 0·5 per cent. solution of chloride of gold until thoroughly yellow, and then exposed to the light in water acidulated with acetic acid until the gold is thoroughly reduced, which happens in the course of a few days at latest. They are then mounted in acidulated glycerin. Results very uncertain and anything but permanent.

364. LÖWIT'S Method (*Sitzgsber. Akad. Wien*, Bd. lxxi, 1875, p. 1).—The following directions are from FISCHER'S paper on the corpuscles of Meissner (*Arch. mik. Anat.*, xii, 1875, p. 366).

Small pieces of *fresh* skin are put into *dilute formic acid* (one volume of water to one of the acid of 1·12 sp. gr.), and remain there until the epidermis peels off. They then are put for fifteen minutes into gold chloride solution ($1\frac{1}{2}$ per cent. to 1 per cent.), then for twenty-four hours into dilute formic acid (1 part of the acid to 1–3 of water), and then for twenty-four hours into undiluted formic acid. (Both of these stages are gone through in the dark). Sections are then made and mounted in dammar or glycerin. Successful preparations show the nerves alone stained.

365. RANVIER'S **Formic Acid Method** (*Quart. Journ. Mic. Sci.* [N.S.], lxxx, 1880, p. 456).—The tissues are placed in a *mixture of chloride of gold and formic acid* (four parts of 1 per cent. gold chloride to one part of formic acid) which has been boiled and allowed to cool (RANVIER'S *Traité,* p. 826). They remain in this until thoroughly impregnated (muscle twenty minutes, epidermis two to four hours) ; reduction is affected either by daylight in acidulated water, or in the dark in dilute formic acid (one part of the acid to four parts of water).

366. RANVIER'S **Lemon-juice Method** (*Traité,* p. 813).—RANVIER finds that of all acids lemon juice is the least hurtful to nerve-endings. He therefore soaks pieces of tissue in fresh lemon juice until they become transparent (five or ten minutes in the case of muscle). They are then rapidly washed in water, brought for about twenty minutes into 1 per cent. gold chloride solution, washed again in water, and brought into a mixture of 50 c.c. of distilled water and two drops of acetic acid. They are exposed to the light for twenty-four to forty-eight hours. The preparations thus obtained are good for immediate study, but are not permanent, the reduction of the gold being incomplete. In order to obtain perfectly reduced, and therefore permanent, preparations, the reduction should be done in the dark in a few cubic centimetres of dilute formic acid (1 part acid to 4 of water), which takes about twenty-four hours.

367. VIALLANE'S **Osmic Acid Method** (*Hist. et Dév. des Insectes,* 1883, p. 42).—The tissues are treated with osmic acid (1 per cent. solution) until they begin to turn brown, then with 25 per cent. formic acid for ten minutes ; they are then put into solution of chloride of gold of 1 : 5000 (or even much weaker) for twenty-four hours in the dark, then reduced in the light in 25 per cent. formic acid. I find this an excellent method.

KERSCHNER (*Arch. mik. Anat.,* lxxi, 1908, p. 522) puts till brown into a mixture of ten parts 5 per cent. formic acid with one part 2 per cent. osmic acid, washes, puts for two to six hours into 1 per cent. gold chloride in the dark, washes, puts for twelve hours into 25 per cent. formic acid in the dark and then for twenty-four in the light, and mounts in 50 per cent. glycerin with 1 per cent. of formol.

368. Other Methods.—The numerous other methods that have been proposed differ from the foregoing partly in respect of the solutions used for impregnation, but chiefly in respect of details imagined for the purpose of *facilitating the reduction* of the gold.

Thus Bastian employed a solution of gold chloride of a strength of 1 to 2000, acidulated with HCl (1 drop to 75 c.c.), and reduced in a mixture of equal parts of formic acid and water *kept warm.*

Hénocque (*Arch. de l'Anat. et de la Physiol.*, 1870, p. 111) impregnates in a 0·5 per cent. solution of gold chloride, washes in water for twelve to twenty-four hours, and reduces in a nearly saturated solution of *tartaric acid* at a temperature of 40° to 50° C. Reduction is effected very rapidly, sometimes in a quarter of an hour.

Hoyer (*Arch. mik. Anat.*, ix, 1873, p. 222) says that the double chloride of *gold and potassium* has many advantages over the simple gold chloride. He impregnates in solutions of 0·5 per cent. strength, and reduces in water containing one or two drops of a *pyrogallic acid* developing solution, such as is used in photography, or in a warm concentrated solution of tartaric acid, at the temperature of an incubating stove.

I have myself used the double chloride of *gold and sodium* with good results.

Ciaccio (*Journ. de Microgr.*, vii, 1883, p. 38) prefers the double chloride of *gold and cadmium.*

Flechsig (*Die Leitungsbahnen in Gehirn*, 1876; *Arch. Anat. u. Phys.*, 1884, p. 453) reduces in a 10 per cent. solution of *caustic soda.*

Nesteroffsky treats impregnated preparations with a drop of *ammonium sulphide,* and finishes the reduction in glycerin (quoted from Gierke's *Färberei z. mik Zwecken*).

Bohm reduces in *Pritchard's solution*—amyl alcohol, 1; formic acid, 1; water, 98.

MANFREDI (*Arch. per le Sci. med.*, v, No. 15) puts fresh tissues into gold chloride, 1 per cent., for half an hour; then *oxalic acid*, 0·5 per cent., in which they are warmed in a water-bath to 36°. Mount in glycerin. Sunny weather is necessary.

BOCCARDI (*Lavori Instit. Fisiol. Napoli*, 1886, i, p. 27; *Journ. Roy. Mic. Soc.*, 1888, p. 155) recommends oxalic acid of 0·1 per cent. or of 0·25 to 0·3 per cent., or a mixture of 5 c.c. pure formic acid, 1 c.c. of 1 per cent. oxalic acid, and 25 c.c. of water, reducing in the dark not longer than two to four hours.

KOLOSSOW (*Zeit. wiss. Mik.*, v, 1888, p. 52) impregnates for two or three hours in a 1 per cent. solution of gold chloride *acidulated* with 1 per cent. of HCl, and reduces for two or three days in the dark in a 0·01 per cent. to 0·02 per cent. solution of *chromic acid*.

GEBERG (*Intern. Monatsschr.*, x, 1893, p. 205) states that previous treatment of tissues for twenty-four hours with *lime-water* (ARNSTEIN's method) greatly helps the reduction.

BERNHEIM (*Arch. Anat. Phys., Phys. Abth.*, 1892, Supp., p. 29) adds to LÖWIT's dilute formic acid a piece of *sulphite of sodium* (must be fresh and smell strongly of sulphurous acid).

Dr. LINDSAY JOHNSON writes to me that besides the "sunning" of the impregnating solution recommended above (§ 349), the gold should be carefully *acidulated with a neutral acetate* or formiate, or acetic or formic acid, at least twenty-four hours before using; and then afterwards the tissue must be washed until no reaction occurs to test-paper.

APÁTHY (*Mikrotechnik*, p. 173; *Mitth. Zool. Stat. Neapel*, xii, 1897, pp. 718—728) lays stress on the necessity of having the objects *thoroughly penetrated by light* from all sides during the process of reduction. Objects, therefore, should always be so thin that light can readily stream through them. He impregnates for a few hours in 1 per cent. gold chloride (§ 361) in the dark, then brings the objects, *without washing out with water*, the gold solution being just superficially mopped up with blotting-paper, into 1 per cent. formic acid. They are to be set up in this, in a tube or otherwise, so that the light *may come through them* *from all sides*, and exposed to diffused daylight in summer, or direct sunlight in winter, for six to eight hours *without a break*. They must *not be moved about* more than can be helped in the acid. If the acid becomes brown it may be changed for fresh. The temperature of the acid should not be allowed to rise over 20° C., whence direct sunlight is to be avoided during the summer. He mounts in glycerin or his syrup (§ 343). He finds such preparations *absolutely permanent*.

Post-Impregnation.

369. GERLACH'S **Method** (STRICKER'S *Handb.*, 1872, p. 678): Spinal cord is hardened for fifteen to twenty days in a 1 to 2 per cent. solution of bichromate of ammonia. Thin sections are made and thrown into a solution of 1 part of double chloride of gold and potassium to 10,000 parts water, which is very slightly acidulated with HCl, and after ten to twelve hours are washed in hydrochloric acid of 1 to 2 : 3000 strength, then brought for ten minutes into a mixture of 1 part HCl to 1000 parts of 60 per cent. alcohol, then dehydrated and mounted in balsam.

(See further, for Nerve Centres, under "Nervous System.")

370. GOLGI (*Mem. Accad. Torino* [2], xxxii, 1880, p. 382) puts tissues previously hardened in 2 per cent. solution of bichromate of potash for ten to twenty minutes into 1 per cent. solution of arsenic acid, then into ½ per cent. solution of chloride of gold and potassium for half an hour, washes in water, and reduces in sunlight in 1 per cent. arsenic acid solution, which is changed for fresh as fast as it becomes brown. Mount in glycerin. Sunny weather is necessary.

371. APÁTHY'S **Method** (*Zeit. wiss. Mik.*, x, 1893, p. 349; *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 729). The material to be used must have been fixed either in sublimate or in a mixture of equal parts of saturated solution of sublimate in 0·5 per cent. salt solution and 1 per cent. osmic acid (this more particularly for Vertebrates). The material should be imbedded *as quickly as possible*, either in paraffin or in celloidin. Sections are made and fixed on slides, and after the usual treatment with iodine, etc., are either put into distilled water for from two to six hours, or are rinsed in water, treated for one minute with 1 per cent. formic acid, and again well washed with water.

They are then put for twenty-four hours, or at least overnight, into the gold-bath, which is preferably 1 per cent. gold chloride (see § 361), but may be weaker, down to 0·1 per cent., after which they are just rinsed with water or superficially dried with blotting-paper. The slides are then set up on end in a sloping position, the sections looking downwards, so that precipitates may not fall on them, in glass tubes filled with 1 per cent. formic acid. The tubes are then exposed to light until the gold is reduced, *as directed in* § 368 *sub. fin.*

I seem to have found it advantageous to reduce in weak solution of *formaldehyde*, either with or without formic acid.

Szütz (*Zeit. wiss. Mik.*, xxix, 1912, p. 292) reduces as Apáthy for one day, then rinses and puts back for the night into the gold, then for the next day again into the formic acid.

372. Impregnation of Marine Animals.—For some reason the tissues of marine animals do not readily impregnate with gold in the fresh state. It is said by Fol that impregnation succeeds better with spirit specimens.

373. Preservation of Impregnated Preparations.—Preparations may be mounted either in balsam or in acidulated glycerin (1 per cent. formic acid).

Theoretically they ought to be permanent if the reduction of the metal has been completely effected, but they are very liable to go wrong through after-blackening. Ranvier states that this can be avoided by putting them for a few days into alcohol, which he says possesses the property of stopping the reduction of the gold.

Blackened preparations may be *bleached* with cyanide or ferricyanide of potassium. Redding employs a weak solution of ferricyanide, Cybulsky a 0·5 per cent. solution of cyanide.

Preparations may be double-stained with the usual stains (safranin being very much to be recommended), but nuclei will only take the second stain in the case of negative impregnation.

Other Metallic Stains.

374. Osmic Acid and Pyrogallol.—This method was first published by me in 1887 (*La Cellule*, iv, p. 110). It consists in putting tissues that have been treated with osmic acid into a weak solution of pyrogallol, in which they quickly turn greenish black, sometimes much too much so.

Hermann (*Arch. mik. Anat.*, xxxvii, 4, 1891, p. 570) put platino-aceto-osmic material hardened in alcohol for twelve to eighteen hours into raw *pyroligneous* acid. This acid ought (*Ergebnisse der Anat.*, ii, 1893, p. 28) to be as raw as possible, and to be of a dark brown colour and evil-smelling. (The stain obtained in this way is *not* due to a mere reduction of the osmic acid, but also to coloration by the brown pyroligneous acid; for Hermann has obtained the same stain with sublimate material, or alcohol material (*op. cit.*, i, 1891 [1892], p. 7).

I find this gives much better results than the pure osmic acid process, but not the best possible. I now proceed as follows:

Hermann or Flemming material is brought *in bulk, directly after fixing,* into a weak aqueous solution of *pyrogallol.* The tissues may remain in it for twenty-four hours, but for small objects an hour or less is sufficient. An *alcoholic* solution of pyrogallol *may* be taken if desired. Rawitz (*Lehrbuch*, p. 60) takes 20 per cent. aqueous sol. of tannin.

There is thus obtained a black stain, which is at the same time a plasma stain and a nuclear stain, chromatin being so far stained that it is not necessary to have recourse afterwards to a special chromatin stain. With Invertebrates it sometimes gives very elegant differentiations of nervous tissue. It is a *very easy* method, and if pyrogallol be used a very *safe* one (with pyroligneous acid not so safe).

If it be desired to add a chromatin stain, I greatly recommend safranin (stain very strongly, twenty-four hours at least, and start the extraction with acid alcohol).

This method has been attributed to VON MAEHRENTHAL. See also under "Nervous System" modifications of this method by AZOULAY and HELLER and GUMPERTZ; also one by KOLOSSOW (*Zeit. wiss. Mik.*, ix, 1892, p. 38, and ix, 1893, p. 316).

375. **Perchloride of Iron.**—This reagent, introduced by POLAILLON (*Journ. de l'Anat.*, iii, 1866, p. 43), sometimes gives useful results, especially in the study of peripheral nerve-ganglia, in which it stains the nervous tissue alone, the connective tissue remaining colourless.

The HOGGANS proceed as follows (*Journ. Quekett Club*, 1876; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 358):—The tissue (having been first fixed with silver nitrate, which is somewhat reduced by a short exposure to diffused light) is dehydrated in alcohol, and treated for a few minutes with 2 per cent. solution of perchloride of iron in spirit; then with a 2 per cent. solution of pyrogallic acid in spirit, and in a few minutes more, according to the depth of tint required, may be washed in water and mounted in glycerin.

FOL fixes in perchloride (§ 80) and treats for twenty-four hours with alcohol containing a trace of gallic acid.

POLAILLON (*loc. cit.*) reduces in tannic acid.

The method is not applicable to chromic objects.

GOLODETZ and UNNA (*Monats. prakt. Derm.* xlviii, 1909, p. 153) put sections of skin for 5 minutes into fresh mixture of 1 per cent. perchloride of iron and 1 per cent. sol. of ferricyanide of potassium. See also UNNA and GOLODETZ, *ibid.*, xlix, 1909, p. 97.

ROOSEVELT (*Med. Rec.*, ii, 1887, p. 84; *Journ. Roy. Mic. Soc.*, 1888, p. 157) employs a stain composed of 20 drops of saturated solution of iron sulphate, 30 grms. water, and 15 to 20 drops pyrogallic acid.

376. **Palladium Chloride** (see SCHULZE, § 77). **Prussian Blue** (see LEBER, *Arch. Ophthalm.*, xiv, p. 300; RANVIER, *Traité*, p. 108). **Cupric Sulphate** (see LEBER, *ibid.*). **Lead Chromate** (see LEBER, *ibid.*). **Sulphides** (see LANDOIS, *Centralb. med. Wiss.*, 1885, No. 55; and GIERKE, in *Zeit. wiss. Mik.*, i, 1884, p. 497). **Molybdate of Ammonia** (MERKEL; KRAUSE) (see GIERKE, *ibid.*, i, 1884, p. 96). **Oxychloride of Ruthenium** (NICOLLE and CANTACUZÈNE) (see *Ann. Inst. Pasteur*, vii, 1893, p. 331). **Ruthenium Red** (Ruthenium Sesquichloride) (EISEN, *Zeit. wiss. Mik.*, xiv, 1897, p. 200; in my hands totally useless). **Oxide of Manganese** (GOLODETZ and UNNA, *Monats. prakt. Derm.*, xlviii, 1909, p. 151).

CHAPTER XVIII.

OTHER STAINS AND COMBINATIONS.

377. Kernschwarz (PLATNER, *Zeit. wiss. Mik.*, iv, 1887, p. 350).—A black liquid on sale by Grübler and Hollborn. MAYER (*Grundzüge*, LEE & MAYER, 1st ed., p. 202) finds that it contains iron, combined with some gallic acid. I use it as follows:

Sections (I have not tried material in bulk) are fixed on slides and treated with Kernschwarz until the required depth of stain is obtained, which will be from a few minutes to twenty-four hours, according to the material.

There is obtained a black or neutral-tint stain, which is either a pure chromatin stain, or at the same time a plasma stain. If overstaining should have occurred, the stain is easily differentiated by means of any weak acid, either in water or alcohol. PLATNER took alkalies, preferably carbonate of lithia, for differentiation.

It may be well, if a good plasma stain has been obtained, to after-stain for twenty-four hours with safranin, followed by differentiation in either neutral or acid alcohol, and clove oil. The stain is perfectly permanent in balsam, and is stated to be a good one for preparations that it is desired to photograph.

I greatly recommend this stain, which is safe and easy. The combination with safranin gives a better chromatin stain than safranin alone.

378. Brazilin, the colouring matter of Brazilian redwood or Pernambuco wood, has been recommended by EISEN (*Zeit. wiss. Mik.*, xiv, 1897, p. 198), and HICKSON (*Nature*, lxii, 1900, p. 589, and *Quart. Journ. Mic. Sci.*, 1901, p. 469). MAYER (*Grundzüge*, p. 203) finds that, in alum solution, it gives a stain similar to that of hæmatein, but much weaker, *Iron-Brazilin* (HICKSON, *Quart. Journ. Micr. Sci.*, xliv, 1901, p. 470) is better. Sections are mordanted for 1 to 3 hours in 1 per cent. sol. of iron alum in alcohol of 70 per cent. (made by dissolving 1 grm. of the salt in 23 c.c. of water, warm, and adding 77 c.c. of 90 per cent. alcohol after cooling), rinsed with alcohol, and put for 3 to 16 hours into 0·5 per cent. sol. of Brazilin in alcohol of 70 per cent.

379. **Orchella (Orseille)** see WEDL (*Arch. path. Anat.*, lxxiv, p. 143); and FOL (*Lehrb.*, p. 192), and early editions of this work.

380. **Orcein** (ISRAEL, *Virchow's Archiv*, cv, 1886, p. 169; and *Prakticum der path. Hist.*, 2 Aufl., Berlin, 1893, p. 72) is a dye obtained from the lichen, *Lecanora parella*, and is not to be confused with *orcin*, another derivative of the same lichen. It is said to unite in itself the staining properties of the basic and acid stains, and also the combination of two contrast colours. Israel stains sections in a solution containing 2 grms. of orcein, 2 grms. of glacial acetic acid, and 100 c.c. of distilled water, washes in distilled water, and passes rapidly through absolute alcohol to thick cedar oil, in which the preparations remain definitely mounted. Nuclei blue, protoplasm red.

See also "Connective Tissues" in Part II, and LAURENT, *Zeit. wiss. Mik.*, xiii, 1896, p. 302; RUZIČKA, *ibid.*, xiv, 1898, p. 455; and WOLFF, *ibid.*, xix, 1903, p. 488.

381. **Purpurin**, see RANVIER'S *Traité technique*, p. 280; DUVAL'S *Précis de Technique histologique*, p. 221; and GRENACHER'S formula in *Arch. Mik. Anat.*, xvi, 1879, p. 470. A very weak stain.

382. **Indigo.**—Indigo is employed in histology in the form of solutions of so-called indigo carmine, or sulphindigotate of soda or potash. The simple aqueous solution gives a diffuse stain, but is of use when employed in conjunction with carmine, see below.

Thiersch's Oxalic Acid Indigo-carmine (see *Arch. mik. Anat.*, i, 1865, p. 150).

383. **Other Vegetal Dyes.**—See *early editions*. Those recommended by CLAUDIUS (*Zeit. wiss. Mik.*, xvii, 1900, p. 52) are superfluous.

Carmine Combinations.

384. **Seiler's Carmine followed by Indigo-Carmine** (*Am. Quart. Mic. Journ.*, i, 1879, p. 220).—Stain in borax-carmine, wash out with HCl alcohol, wash out the acid, and after-stain in an *extremely dilute* alcoholic solution of indigo-carmine (two drops of saturated aqueous solution added to an ounce of alcohol and filtered).

I find this method gives good results with sections, but not if it be attempted to stain in bulk.

385. Merkel's Carmine and Indigo-Carmine in One Stain (MERKEL, *Unters. anat. Anst. Rostock*, 1874; *Month. Mic. Journ.*, 1877, pp. 242 and 317).

Also NORRIS and SHAKESPEARE, *Amer. Journ. Med. Sci.*, January, 1877; MERKEL, *Mon. Mic. Journ.*, 1877, p. 242; MARSH, *Section Cutting*, p. 85; BAYERL, *Arch. Mik. Anat.*, xxiii, 1885, pp. 36, 37; MACALLUM, *Trans. Canad. Instit.*, ii, 1892, p. 222; *Journ. Roy. Mic. Soc.*, v, 1892, p. 698.

386. MAYER'S Carmalum (or Hæmalum) and Indigo-Carmine in one Stain.—MAYER (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 320) obtains very good results by taking a solution of 0·1 grm. of indigo-carmine in 50 c.c. of distilled water, or 5 per cent. alum solution, and combining it with from four to twenty volumes of carmalum or hæmalum.

387. Carmine and Picro-Indigo-Carmine (RAMÓN Y CAJAL, *Rev. de Cienc. med.*, 1895; CALLEJA, *Rev. trim. Microgr.*, ii, 1897, p. 101; *Zeit. wiss. Mik.*, xv, 1899, p. 323).—For use after a carmine stain, RAMÓN takes a solution of 0·25 grm. of indigo-carmine in 100 grms. saturated aqueous solution of picric acid. Stain (*sections*) for five to ten minutes, wash in weak acetic acid, then in water, then remove the excess of picric acid with absolute alcohol, clear and mount.

RAMÓN also (*Elementos de Histologia*, 1897; quoted from *La Cellule*, xix, 1901, p. 212) employs the picro-indigo mixture after **Magenta**; stain strongly in saturated solution of magenta, rinse in water until no more colour comes away, and pass into the indigo mixture. See also BORREL, *Ann. Inst. Pasteur*, 1901, p. 57, or LEE et HENNEGUY, *Traité*, p. 268.

388. Carmine and Anilin Blue (or Bleu Lumière, or Bleu de Lyon) (DUVAL, *Précis de Technique Microscopique*, 1878, p. 225).—Stain with carmine; dehydrate, and stain for a few minutes (ten minutes for a section of nerve-centres) in a solution of ten drops of saturated solution of anilin blue in alcohol to 10 grms. of absolute alcohol. Clear with turpentine, without further treatment with alcohol, and mount in balsam.

Other authors recommend, instead of anilin blue, bleu de Lyon, dissolved in 70 per cent. alcohol acidulated with acetic acid (MAURICE and SCHULGIN), or bleu lumière.

The solutions of both these colours should be extremely dilute for sublimate material, but strong for chrom-osmium material. It is possible to use them for staining in bulk.

BAUMGARTEN (*Arch. mik. Anat.*, xl, 1892, p. 512) stains sections (of material previously stained in borax-carmine) for twelve hours in a 0·2 per cent. solution of bleu de Lyon in absolute alcohol, and washes out for about half that time before mounting in balsam. He recommends the process for cartilage and nerve-centres.

389. Carmine and Malachite Green.—MAAS (*Zeit. wiss. Zool.*, l, 4, 1890, p. 527) recommends borax-carmine followed by weak alcoholic solution of malachite green, with a final washing out with stronger alcohol.

390. Carmine and Picro-nigrosin (PIANESE). See *Journ. Roy. Mic. Soc.*, 1892, p. 292.

391. Carmine and Picric acid. See § 298.

Hæmatein or Hæmatoxylin Combinations.

392. Hæmatoxylin and Picric Acid.—See § 298.

393. Hæmatoxylin and Eosin.—This popular combination gives results that are æsthetically beautiful, but (for most objects) is not so useful as many others, the eosin lacking in electivity. Objects may be stained with hæmatoxylin (either in the mass or as sections) and the sections stained for a few minutes in eosin. I think it is better to take the eosin weak, though it has been recommended (STÖHR, see *Zeit. wiss. Mik.*, i, 1884, p. 583) to take it saturated. Either aqueous or alcoholic solutions of eosin may be used.

HICKSON (*Quart. Journ. Mic. Sci*, 1893, p. 129) stains sections for one hour in a strong solution of eosin in 90 per cent. alcohol, washes with alcohol, and stains for twenty minutes in a weak solution of hæmatoxylin.

This method is most particularly recommendable for embryological sections, as vitellus takes the eosin stain energetically, and so stands out boldly from the other germinal layers in which the blue of the hæmatoxylin dominates.

See also LIST (*Zeit. wiss. Mik.*, ii, 1885, p. 148) ; BUSCH

(*Verh. Berl. Phys. Gses.*, 1887); GIERKE (*Zeit. wiss. Mik.*, i, 1884, p. 505).

Sections should be very well washed before being passed from eosin into hæmatoxylin or the reverse, as eosin very easily precipitates hæmatoxylin.

For the complicated and superfluous *mixtures* of RENAUT and of EVERARD, DEMOOR and MASSART, see FOL'S *Lehrbuch*, p. 196, *Ann. Inst. Pasteur*, vii, 1893, p. 166, or *early editions*.

394. Hæmatoxylin and Congo.—See § 305.

395. Hæmatoxylin and Safranin.—RABL (*Morph. Jahrb.*, x, 1884, p. 215) stained *very lightly* with *very dilute* DELAFIELD'S hæmatoxylin for twenty-four hours, then for some hours in (PFITZNER'S) safranin and washed out with pure alcohol. The plasma stain is here given by the hæmatoxylin.

Similarly REGAUD, *Verh. Anat. Ges.*, xiv, 1900, p. 112.

FOÀ (*Festschr. Virchow*, 1891, p. 481) stains in a *mixture* of 25 c.c. of Böhmer's hæmatoxylin, 20 of 1 per cent. solution of safranin, and 100 of water for one to three minutes.

396. Hæmatoxylin and Säurefuchsin.—Stain first with iron hæmatoxylin or hæmalum, then stain (sections) in 0·5 per cent. aqueous solution of Säurefuchsin, dehydrate and mount.

397. Hæmatoxylin and Säurefuchsin and Orange.—Proceed as above, using for the second stain the following mixture: Säurefuchsin, 1 grm.: orange, 6 grms.; rectified spirit, 60 c.c.; water, 240 c.c. (from SQUIRE'S *Methods and Formulæ*, p. 42). Using orange G. (not mentioned by SQUIRE), I have had very good results.

The method of CAVAZZANI (*Riforma Med.*, Napoli, 1893, p. 604; *Zeit. wiss. Mik.*, xi, 3, 1894, p. 344) is far too complicated.

398. Hæmatoxylin and Picro-Säurefuchsin (VAN GIESON, *New York Med. Journ.*, 1889, p. 57; quoted from MOELLER, *Zeit. wiss. Mik.*, xv, 2, 1898, p. 172, which see for further details). Proceed as above, using for the second stain the picro-

Säurefuchsin mixture, § 299. The second stain must not be too prolonged.

WEIGERT (*Zeit. wiss. Mik.*, xxi, 1904, p. 1) stains first in his iron-hæmatoxylin mixture (§ 244), rinses in water, and stains for a short time in his picro-Säurefuchsin (§ 299), rinses, dehydrates with 90 per cent. alcohol, and clears with carbolic acid-xylol mixture (§167).

CHAPTER XIX.

EXAMINATION AND PRESERVATION MEDIA.

399. Introductory.—I comprehend under this heading all the media in which an object may be examined to advantage.

All preservative media may be used for mounting, though the only media that will afford an *absolutely sure* preservation of soft tissues are the resinous ones.

400. Refractive Indices of Examination Media.—An examination medium should be of such a *refractive index* as to afford a due degree of *visibility* of *colourless* (*unstained*) elements. The visibility of these is inversely as their transparency when penetrated by the medium. It is directly proportional to the *difference* between the *refractive indices* of the object and of the medium in which it is mounted. The greatest *transparency* is obtained when the refraction of the medium is the same as that of the tissue elements. Media having a lower index than that of the tissues give diminished transparency, but greater *visibility*. Media having a higher index than that of the tissues give great transparency, but *diminished visibility* of (unstained) details. Now the index of refraction of most tissue elements, after fixation and dehydration, is somewhat higher than that of Canada balsam; so that media of the greatest clearing power (*i. e.* giving the greatest transparency) must be looked for amongst reagents having an index superior to that of balsam, whilst for enhanced visibility of detail we must employ less refractive media, such as castor oil, glycerin, or water.

The following short list, extracted from Behrens' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten*, Braunschweig, 1892, p. 42, and other sources, may be useful as a guide to the *optical effects* of various media. The figures give the *approximate indices of refraction.* They should be accepted with some caution, on account of the variability of samples. The figures given for balsam refer evidently to the resin in the solid state and not to the solutions used for mounting, which are certainly much lower, according to the lower index of the solvent.

Air	1·000
Methyl alcohol	1·323
Distilled water	1·336
Sea water	1·343
Solution of white of egg	1·350
Absolute alcohol	1·367
Acetate of potash, saturated aqueous sol.	1·370
Glycerine with an equal quantity of water	1·397
Chloride of calcium, 90 per cent. in water	1·411
Glycerine, Price's	1·460
Oil of bergamot	1·464
Paraffinum liquidum	1·471
Olive oil	1·473
Oil of turpentine	1·473
Glycerine, "concentrated"	1·473
Gilson's Baume au Camsal	1·478
Gilson's Euparel	1·483
Terpinol	1·484
Castor oil	1·490
Xylol	1·497
Cedar-wood oil, not thickened	1·510
Crown glass	1·518
Cedar-wood oil, thickened	1·520
Gum damar	1·520
Xylol balsam	1·524
Oil of lemons	1·527
Oil of cloves	1·533
Canada balsam (solid)	1·535
Creasote	1·538
Colophonium	1·545
Carbolic acid	1·549
Oil of anise seed	1·557
Oil of cinnamon (or cassia)	1·567
Anilin oil	1·580
Sulphide of carbon	1·630
Tolu balsam	1·640
Monobromide of naphthalin	1·660
Solution of sulphur in sulphide of carbon	1·750

It will be seen that cedar oil has nearly the index of crown glass (this is true of the oil in the thick state to which it is brought by exposure to the air—not of the new, thin oil, which is less highly refractive); it therefore clears to about the same extent as Canada balsam. Clove oil has a much higher index, and therefore clears more than balsam; cinnamon oil higher still. Turpentine and bergamot oil have much lower indices, and therefore clear less.

Watery Media.

401. Isotonic and "Indifferent" Liquids.—The old distinction of "Indifferent" liquids, and those which have some action on tissues, appears to be misleading more than helpful; for *no* medium is without action on tissues except the plasma with which they are surrounded during the life of the organism;* and this plasma itself is only "indifferent" whilst all is *in situ*; as soon as a portion of tissue is dissected out and transferred to a slide in a portion of plasma the conditions become artificial.

Water may be employed for the examination of structures that have been *well fixed*; but this is by no means applicable to the examination of fresh tissues. It is very far from being an "indifferent" liquid; many tissue elements are greatly changed by it (nerve-end structures, for instance), and some are totally destroyed by its action if prolonged (for instance, red-blood corpuscles).

In order to render it inoffensive to fresh tissues it must have dissolved in it substances of similar diffusibility to those of the liquids of the tissue, so as to prevent the occurrence of osmosis, to which process the destructive action of pure water is mainly due. Now cell contents are a mixture of colloids and crystalloids; consequently, in order to reduce osmotic processes to a minimum, it is necessary that the examination medium contain a due proportion of both crystalloids and colloids. By adding, for instance, white of egg to salt solution this end may be in some measure attained; and, as a matter of fact, the liquids recommended as "indifferent" are generally found to contain both crystalloids and colloids. Liquids thus composed, in which tissue-elements are in osmotic equilibrium—that is, neither swell nor shrink—are said to be *isotonic* to the tissues; whilst those in which they shrink are called *hypertonic*, and those in which they swell *hypotonic*. Solutions of common salt, in different concentrations, form the base of the most commonly employed isotonic liquids. For marine Invertebrates, sea-water is generally isotonic.

402. Salt Solution ("normal salt solution," "physiological salt solution").—0·75 per cent. sodium chloride in water. CARNOY recommends the addition of a trace of osmic acid.

RINGER'S solution, much used in physiology, consists of sodium chloride 0·8 parts, calcium chloride 0·02, potassium chloride 0·02, sodium bicarbonate 0·02 and water 100 (with or without 0·1 dextrose).

According to Locke (*Boston. Med. Surg. Journ.*, 1896, p. 514) there should be added to salt solution (which to be isotonic should contain, according to Hamburger, 0·9 to 1 per cent. of salt)—0·01 per cent. chloride of potassium, and 0·02 per cent. chloride of calcium, in order to obtain an "indifferent" liquid.

Malassez (*C. R. Soc. Biol.*, iii, 1896, pp. 504 and 511) takes for erythrocytes about 1 per cent. sodium chloride.

Dekhuysen (*Onderz. Phys. Lab. Leiden.*, 1900, p. 149) takes for blood of *Rana* 0·8 per cent.

For *Selachians*, Muskens (*Tijd. Nederb. Dierk. Ver.*, 1894, p. 314) finds $2\frac{1}{4}$ per cent. right; and Rodin (*Comptes Rend.*, 1900, p. 1009) 1·5 to 2·6 per cent., according to the species.

Engelmann (*Deutsch. med. Wochenschr.*, xxix, 1903, p. 64), finds that 0·9 per cent. is isotonic with human blood-serum, and 0·64 per cent. for red blood corpuscles of the frog.

Kronecker's "Artificial Serum" (from Vogt et Yung *Traité. d'Anat. Comp. Prat.*, p. 473) consists of common salt 6 parts, caustic soda 0·06, distilled water 1000.

Böhm und Oppel (*Taschenbuch*, 3 Aufl., p. 19) take carbonate of soda instead of caustic soda.

403. Pictet's **Liquid** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 89).—5 to 10 per cent. solution of chloride of manganese. These proportions are for *marine* animals, and for terrestrial animals will generally be found much too high. For these from 1 per cent. to 3 per cent. will be nearer the mark. I find this liquid excellent.

404. Aqueous Humour, Simple White of Egg.—Require no preparation beyond filtering. They may be iodised if desired (see next §), or mixed with salt solution.

405. Iodised Serum.—Max Schultze (*Virchow's Archiv*, xxx, 1864, p. 263). I take the following from Ranvier (*Traité*, p. 76).

The only serum that gives really good results is the amniotic liquid of mammals. Flakes of iodine are added to it, and the flask frequently agitated for some days. The flask should have a wide bottom, so that the serum may form only a shallow layer in it.

Another method is as follows: Serum is mixed with a large proportion of tincture of iodine; the precipitate that forms is removed by filtration, and there remains a strong solution of iodine in serum. This should be kept in stock, and a little of it added every two or three days to the serum that is intended for use. In general for maceration purposes a serum of a pale brown colour should be employed.

406. **Artificial Iodised Serum** (FREY, *Das Mikroscop*, 6 Aufl., 1877, p. 75).—Distilled water 270 grms., white of egg 30, sodium chloride 2·5. Mix, filter, and add tincture of iodine.

407. **MIGULA'S Glycerized Blood-serum** (see the paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 172).

408. **Chloride of Calcium** (HARTING, *Das Mikroskop*, 2 Aufl., p. 297).—The aqueous solution, either saturated or diluted with 4 to 8 parts of water, has a low refractive index and does not dry up.

409. **Acetate of Potash** (MAX SCHULTZE, *Arch. mik. Anat.*, vii, 1872, p. 180).—A nearly saturated solution in water. The index of refraction is lower than that of glycerin.

410. **Syrup.**—A good strength is equal parts of loaf sugar and water. Dissolve by boiling. To preserve it from mould, chloral hydrate may be dissolved in it (1 to 5 per cent.)—I have used as much as 7 per cent., and found no disadvantage—or carbolic acid (1 per cent.).

It may be used as a mounting medium, but there is always risk of the sugar crystallising out.

FABRE-DOMERGUE (*Bull. Soc. Philomath*, ix, 1899, p. 115) dissolves 200 parts of suger in 400 of water, and adds 1 part of formaldehyde, and camphor to saturation.

411. **Chloral Hydrate.**—5 per cent. in water (LADOWSKY, *Arch. f. mik. Anat.*, 1876, p. 359).

Or, 2·5 per cent. in water (BRADY, *British Copepods*).

Or, 1 per cent. in water (MUNSON, *Journ. Roy. Mic. Soc.*, 1881, p. 847).

Mercurial Liquids.

(*I give these as examination media only, not as permanent mounting media. Media containing sublimate always end by making tissues granular.*)

412. Gilson's **Fluid** (Carnoy's *Biologie cellulaire*, p. 94).

Alcohol of 60 per cent.	60 c.c.
Water	30 ,,
Glycerin	30 ,,
Acetic acid (15 parts of the glacial to 85 of water)	2 ,,
Bichloride of mercury	0·15 grm.

413. Gage's **Albumen Fluid** (*Zeit. f. wiss. Mik.*, 1886, p. 223).

White of egg	15 c.c.
Water	200 ,,
Corrosive sublimate	0·5 grm.
Salt	4 grms.

Mix, agitate, filter, and preserve in a cool place. Recommended for the study of red blood-corpuscles and ciliated cells.

414. Pacini's **Fluids** (*Journ. de Mik.*, iv, 1880; *Journ. Roy. Mic. Soc.*, [N.S.] ii, 1882, p. 702, and early editions of this work).—Antiquated and superfluous. They consist essentially of corrosive sublimate of from one half to one third per cent. strength, with the addition of a little salt or acetic acid.

415. Goadby's **Fluids** (*Micro. Dict.*, art. "Preservation," or early editions of this work).—Quite unsuited for histological purposes.

Other Fluids.

416. **Chloride and Acetate of Copper** (Ripart et Petit's fluid, see § 90).

417. **Tannin** (Carnoy, *Biol. Cellulaire*, p. 95).—Water 100 grms., powdered tannin 0·40 grm., as an examination medium only.

418. Wickersheimer's **Fluid** (*Zool. Anz.*, 1879, p. 670).—Worthless for histological purposes.

419. **Medium** of Farrants (Beale, *How to Work*, etc., p. 58).—Picked gum arabic 4 ozs., water 4, glycerin 2,

See also the *Micrographic Dictionary*, and A. F. STANLEY KENT, in *Journ. Roy. Mic. Soc.*, 1890, p. 820.

420. Gum and Glycerin Medium (LANGERHANS, *Zool. Anzeig.* ii, 1879, p. 575).

Gummi arab.	5·0
Aquæ	5·0
To which after twelve hours are added—	
Glycerini	5·0
Sol. aquosa acid. corbal. (5·100)	10·0

421. ALLEN'S **Gum and Glycerin.**—Prof. F. J. ALLEN (*in. litt.*). Solution of gum arabic of the consistency of glycerin, strained, and one eighth volume of glycerin and one twentieth of formol gradually incorporated. *Sets hard.*

422. HOYER'S **Gum with Chloral Hydrate or Acetate of Potash** (*Biol. Centralb.*, ii, 1882, pp. 23, 24).—A high 60 c.c. glass with a wide neck is filled two thirds full with gum arabic (in pieces), and then *either* a solution of chloral (of several per cent.) containing 5—10 per cent. of glycerin is added *or* officinal solution of acetate of potash or ammonia. Filter after solution. The solution with chloral is for carmine or hæmatoxylin objects—that with acetate for anilin objects.

423. COLE'S **Gum and Syrup Medium.** See § 183.

424. APÁTHY'S **Gum and Syrup Medium** (see § 343).—This medium sets very hard and may also be used for ringing glycerin mounts.

425. FABRE-DOMERGUE'S **Glucose Medium** (*La Nature*, No. 823, 9 Mars, 1889, supp.).—Glucose syrup diluted to twenty-five degrees of the areometer (sp. gr. 1·1968) 1000 parts, methyl alcohol 200, glycerin 100, camphor to saturation. The glucose is to be dissolved in warm water, and the other ingredients added. The mixture, which is always acid, must be neutralised by the addition of a little potash or soda. It is said to preserve without change almost all *animal pigments*.

426. BRUN'S **Glucose Medium** (from FABRE-DOMERGUE'S *Premiers Principes du Microscope*, 1889, p. 123).—Distilled water 140 parts, camphorated spirit 10, glucose 40, glycerin 10. Mix the water, glucose, and glycerin, then add the spirit, and filter. HENNEGUY informs me that this liquid preserves the colour of preparations stained with anilin dyes, *methyl green included.*

427. **Levulose** is recommended by BEHRENS, KOSSEL u. SCHIEFFERDECKER (*Das Mikroskop, etc.*, 1889). It is uncrystallisable, and preserves well carmine and coal-tar stains (hæmatoxylin stains fade somewhat in it). The index of refraction is somewhat higher than that of glycerin. Objects may be brought into it out of water.

428. AMANN'S **Lactophenol** (from LANGERON, *C. R. Soc. Biol.*, lviii, 1905, p. 750).—Carbolic acid, 20; lactic acid, 20; glycerin, 40; water, 20. For Nematodes, Acarids, etc. Add gradually drop by drop to the water containing the organisms. Not for mounting. Mount in glycerin jelly.

Glycerin Media.

429. **Glycerin.**—Glycerin diluted with water is frequently employed as an examination and mounting medium. Dilution with water is sometimes advisable on account of the increased visibility that it gives to many structures. But for efficacious preservation undiluted glycerin, the strongest that can be procured, should be used (see BEALE, *How to work*, etc.).

For closing glycerin mounts, the edges of the cover should first (after having been cleansed as far as possible from superfluous glycerin) be painted with a layer of *glycerin jelly*; as soon as this is set a coat of any of the usual cements may be applied. See next chapter.

Glycerin dissolves carbonate of lime, and is therefore to be rejected in the preparation of calcareous structures that it is wished to preserve.

430. **Extra-refractive Glycerin.**—The already high index of refraction of glycerin (Price's glycerin, $n = 1{\cdot}46$) may be raised by dissolving suitable substances in it. Thus the refractive index of a solution of chloride of cadmium ($CdCl_2$) in glycerin may be 1·504; that of a saturated solution of sulphocarbolate of zinc in glycerin may be 1·501; that of a saturated solution of SCHERING'S chloral hydrate (in crusts) in glycerin is 1·510: that of iodate of zinc in glycerin may be brought up to 1·56. For further details see *previous editions*, or *Journ. Roy. Mic. Soc.*, ii, 1879, p. 346; iii, 1880, p. 1051; (N.S.), i, 1881, pp. 943 and 366.

431. **Glycerin and Alcohol Mixtures.**—These afford one of the best means of bringing delicate objects gradually from weak into strong glycerin. The object is mounted in a drop of the liquid, and left for a few hours or days, the mount not

being closed. By the evaporation of the alcohol the liquid gradually increases in density, and after some time the mount may be closed, or the object brought into pure glycerin or glycerin jelly.

1. Calberla's Liquid (*Zeit. wiss. Zool.*, xxx, 1878, p. 442).—Glycerin 1 part, alcohol 2, water 3.

2. I strongly recommend the following for very delicate objects:—Glycerin 1 part, alcohol 1, water 2.

3. Hantsch's Liquid.—Glycerin 1 part, alcohol 3, water 2.

4. Jäger's Liquid (Vogt and Yung's *Traité d'Anat. comp. prat.*, p. 16).—Glycerin 1 part, alcohol 1, sea water 10.

Glycerin Jellies.

432. **Glycerin Jellies** have a higher index than pure glycerin, and set hard enough to make luting unnecessary, though it is well to varnish the mount. To use them, you melt a small portion on a slide, introduce the object (previously soaked in water or glycerin), and cover. They seem very plausible, but for delicate work I do not recommend them, and should advise instead either pure glycerin or euparal.

433. Lawrence's **Glycerin Jelly** (Davies, *Preparation and Mounting of Microscopic Objects*, p. 84).—Soak some gelatin for two or three hours in cold water, pour off the superfluous water, and heat until melted. To each fluid ounce of the gelatin, *whilst it is fluid but cool*, he adds a fluid drachm of the white of an egg. Boil until tho albumen coagulates and the gelatin is quite clear, and to each ounce of the solution add 6 drachms of a mixture composed of 1 part of glycerin to 2 parts of camphor water.

434. Brandt's **Glycerin Jelly** (*Zeit. wiss. Mik.*, ii, 1880, p. 69).—Melted gelatin 1 part, glycerin 1½ parts. The gelatin to be soaked in water and melted as above. After incorporating the glycerin, filter through spun glass pressed into the lower part of a funnel. He describes a simple arrangement for keeping the funnel warm during the filtering (see *early editions*). Some drops of carbolic acid should be added.

435. Kaiser's **Glycerin Jelly** has been given § 155.

436. SQUIRE'S **Glycerin Jelly** (SQUIRE'S *Methods and Formulæ*, etc., p. 84).—Soak 100 grms. of French gelatin in chloroform water, drain when soft, and dissolve with heat in 750 grms. of glycerin. Add 400 grms. of chloroform water with which has been incorporated about 50 grms. of fresh egg-albumen; mix thoroughly, and heat to boiling-point for about five minutes. Make up the total weight to 1550 grms. with chloroform water. Filter in a warm chamber.

437. HEIDENHAIN (*Zeit. wiss. Mik.*, xx, 1905, p. 328) takes of gelatin 9 parts, glycerin 7, and water 42, and to the filtrate adds drop by drop 14 parts of absolute alcohol.

438. FISCHER (*ibid.*, xxix, 1912, p. 65) takes 5 grms. of borax disolved in 240 c.c. of water and adds 25 c.c. of glycerine. To this he adds 40 grms. of gelatin, dissolves with heat, and continues to heat gently until the solution has somewhat thickened. This remains fluid at ordinary temperatures.

439. GILSON'S **Chloral Hydrate Jelly** (communicated by GILSON).—1 vol. of gelatin, melted *secundum artem*, and 1 vol. of Price's glycerin. Mix, and add crystals of chloral hydrate until the volume has increased by one half; warm till dissolved. This gives a very *highly refractive* medium.

GEOFFROY, *Journ. de Botan.*, 1893, p. 55 (see *Zeit wiss. Mik.*, ix, 1893, p. 476) dissolves, by the aid of as little heat as possible, 3 to 4 grms. of gelatin in 100 c.c. of 10 per cent. aqueous solution of chloral hydrate.

High Refractive Liquids.

440. STEPHENSON'S **Biniodide of Mercury and Iodide of Potassium** (*Journ. Roy. Mic. Soc.* [N. S.], ii, 1882, p. 167).—A solution prepared by adding the two salts to water until each is in excess; the liquid will then be found to have a refractive index of 1·68. (If [AMANN, *Zeit. wiss. Mik.*, xiii, 1896, p. 21] glycerin be taken instead of water, it rises to 1·78 or 1·80. BEHRENS [*Tabellen*, 1898, p. 71] takes biniodide 65 parts, iodide 50, and water 25. $n = 1·71$.) Any

lower index can be obtained by suitable dilution with water. This fluid is very dense, its specific gravity being 3·02. It is highly antiseptic.

For marine animals a weak solution is probably well adapted, as about a 1 per cent. solution (5 minims to the ounce) will give the specific gravity of sea water.

Covers should be sealed with white wax, and the mounts finished with two or three coatings of gold size and one of shellac.

I have experimented both with strong and with weak solutions. They are not adapted, I find, for the purposes of a *permanent* mounting medium, for the preparations are ruined by a precipitate which forms in the fluid. But as a temporary examination medium I have occasionally found this solution valuable. Its optical properties are wonderful; it allows of the examination of watery tissues, *without any dehydration*, in a medium of refractive index surpassing that of any known resinous medium.

See further details in *early editions*.

441. **Monobromide of Naphthalin.**—See *Journ. Roy. Mic. Soc.*, 1880, p. 1043 (Abbe and Van Heurck), and *Zool. Anz.*, 1882, p. 555 (Max Flesch).

Resinous Media.

442. **Resins and Balsams.**—Resins and balsams consist of a vitreous or amorphous substance held in solution by an essential oil. By distillation or drying in the air they lose the essential oil and pass into the solid state. It is these solidified resins that should be employed for microscopical purposes; for the raw resins always contain a certain proportion of water, which makes it difficult to obtain a clear solution with the usual menstrua, is injurious to the optical properties of the medium and to the preservation of stains. All solutions should therefore be made by heating gently the balsam or resin in a stove until it becomes brittle when cold, and then dissolving in an appropriate menstruum.

Solutions made with volatile menstrua, such as xylol and chloroform, set rapidly, but become rapidly brittle. Solutions made with non-volatile media, such as turpentine, set much less rapidly, and pass much less rapidly into the brittle state.

Turpentine media preserve the *index of visibility* of the preparations much longer than do media made with more volatile menstrua. Preparations made with these often become so transparent in course of time that much fine detail is often lost. (Such mounts may, however, be revivified without removing the cover by putting them for a day or two into a tube of benzol; the benzol penetrates the balsam, and brings it down to a lower refractive index.)

For a permanent mounting medium of somewhat low index I unhesitatingly recommend *Euparal.* For cases in which a still lower index is desired, Gilson's *camsal balsam. Turpentine colophonium* is a safe and excellent medium, but is injurious to alum-hæmatein stains. For these, and in general where a strongly clearing medium is desired, *xylol balsam* is about the most recommendable, though it is not *perfectly* safe, the mounts sometimes developing granules. Seiler's *alcohol balsam* is a fine medium, and perfectly stable. *Oil of cedar* is sometimes useful, it keeps perfectly, and with time it thickens sufficiently to hold the cover in place; or if desired, preparations may be luted with Bell's cement.

443. Canada Balsam.—Prepare with the solid balsam as described last §. The usual menstrua are xylol, benzol, chloroform, and turpentine. Turpentine has the advantages pointed out last §, but the defect that it does not always give a homogeneous solution with Canada balsam, as it does with colophonium. For most purposes *the xylol solution is the best.* If time be an object, a benzol solution should be preferred, as it sets much quicker than the xylol solution. The chloroform solutions become very brown with age, and are *injurious to stains made with tar dyes.* Benzol is good when chemically pure and *free from water.*

Sahli (*Zeit. wiss. Mik.*, ii, 1885, p. 5) dissolves in cedar oil.

Apáthy (*Fauna Flora Golf. Neapel*, xxii, 1909, p. 18) takes balsam 2 parts, cedar oil (immersion) 1, and chloroform 1.

Samples of balsam that are *acid* are frequently met with, and are injurious to some stains. Grübler & Hollborn now prepare a *neutral balsam*, in which Mayer has found that very delicate preparations, that lost colour immediately in any other sort of balsam, have kept perfectly for many months. For a process of neutralising balsam with carbonate of soda or potash see COLUCCI (*Giorn. Ass. Med. Natural Napoli*, vii, 1897, p. 172).

444. SEILER's **Alcohol Balsam** (*Proc. Amer. Soc. Mic.*, 1881, pp. 60–2; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, pp. 126–7).—Dissolve solid balsam in warm absolute alcohol, and filter through absorbent cotton. Objects may be mounted in it *direct from absolute alcohol.* I find it for most purposes admirable. It is *one of the most stable solutions known to me.* Care should be taken not to breathe on it, as this may cause cloudiness.

445. Damar (Gum Damar, or Dammar, or d'Ammar).—The menstrua are the same as for balsam. I find xylol the best. For directions for preparing solutions, by various authors, see *early editions.* After ample experience I am convinced that *not one of these solutions can be depended on for permanent preservation.* Sooner or later, sometimes after a few weeks or days, or it may be only after months or years, granules make their appearance in the mounts.

446. Colophonium.—A solution of pale colophonium in *oil of turpentine* keeps well and gives very good definitions. The solution should not be too thick, as it thickens with age.

This medium drys very slowly (so that ample time is afforded for arranging objects in it). In the winter a slide will take about a month before it will be hard enough to be safe with *oil-immersion lenses;* whereas an alcohol-balsam mount will be dry enough in a couple of days. It injures alum-hæmatein stains; but with these exceptions I find it *a most excellent medium.*

REHM (*Zeit. wiss. Mik.*, ix, 1893, p. 387) dissolves 1 part colophonium in 10 of *benzin.* Solutions in chloroform or xylol are also used by some, see NISSL in *Encycl. mik. Techn.*, ii, p. 274.

447. Venice Turpentine (VOSSELER, *Zeit. wiss. Mik.*, vi, 1889, p. 292, *et seq.*).—Commercial Venice turpentine is mixed in a tall cylinder glass with an equal volume of 96 per cent. alcohol, allowed to stand in a warm place for three or four weeks, and decanted. Preparations may be mounted in this medium direct from absolute alcohol. Celloidin sections can be mounted direct from 96 per cent. Stains keep well, according to VOSSELER, but MAYER finds hæmalum stains fade in it.

SUCHANNEK (*ibid.*, vii, 1896, p. 463) prepares it with equal parts of Venice turpentine and *neutral* absolute alcohol.

448. **Thickened Oil of Turpentine** has been used as a mounting medium by some workers. To prepare it, pour some oil into a plate, cover it lightly so as to protect it from dust without excluding the air, and leave it until it has attained a syrupy consistency.

449. GILSON'S **Sandarac Media** (*La Cellule*, xxiii, 1906, p. 427: the formulæ have not been published, on account of the extreme difficulty of preparation, but the products are on sale by Grübler & Hollborn, even if not listed). There are three of these. They are all of them solutions of gum Sandarac in "Camsal" and other solvents ("Camsal" is a liquid formed by the mutual solution of the two solids salol and camphor).

(1) **Camsal balsam (baume au camsal), propylic alcohol formula**; a mixture of sandarac, camsal, and propylic alcohol, $n = 1{\cdot}478$.

(2) **Camsal balsam, isobutylic alcohol formula**, $n = 1{\cdot}485$.

(3) **Euparal**, a mixture of camsal, sandarac, eucalyptol, and paraldehyde, $n = 1{\cdot}483$. There are two sorts of this, the colourless and the green ("euparal vert"), the latter containing a salt of copper, which *intensifies hæmatoxylin stains.*

Objects may be prepared for mounting in camsal balsam by a bath of propylic or isobutylic alcohol; and for euparal by a bath of the special solvent (supplied by Grübler & Hollborn under the name of "essence d'euparal"). But this is *not necessary.* Objects may *always* be mounted *direct* from absolute alcohol, and even *at a pinch from alcohol of* 70 *per cent.* I myself generally prefer alcohol of 95 per cent. (absolute is dangerously volatile for sections). In difficult cases you may pass through a mixture of the medium and the solvent.

These media *work very kindly,* and do not dry too rapidly. They are *not oxidant,* and preserve delicate stains (perfectly, so far as I know). The mounts seem to keep *perfectly,* without scaling: all of mine, the oldest being eight years old, have kept without the slightest deterioration in any respect.

The primary intention of these media is to spare delicate objects the usual treatment with absolute alcohol and essential oils. But they have another useful property—their *low index of refraction.* I find that that of euparal is just right for most delicate cytological researches, giving just the desired increase of visibility to unstained elements. Thus I frequently find that unstained spindles which are totally invisible in balsam become strongly visible in the most minute details in euparal. The camsal balsam, $n = 1{\cdot}478$, I have also sometimes found valuable, but its index is a little too low for most things, and I generally prefer euparal, which I find I am now using almost as much as balsam. I consider that all the media which have been recommended on the score of a slightly lower index than balsam, such as damar, colophonium, Venice turpentine, castor-oil, are now superseded by these media.

450. **Sandarac** (Lavdowsky, from *Ref. Handbook Med. Sci.*, Supp. p. 438).—Gum sandarac 30 grs., absolute alcohol 50 c.c. Not trustworthy, the mounts scale badly.

451. **Photographic Negative Varnish** (for mounting large sections without cover-glasses).—See Weigert, *Zeit. wiss. Mik.*, iv, 1887, p. 209.

452. **Castor Oil.**—See Grenacher, *Abhandl. naturf. Ges. Halle-a.-S.*, Bd. xvi; *Zeit. wiss. Mik.*, 1885, p. 244. I have not had good results with it.

453. **Terpinol.**—$n = 1{\cdot}484$. See § 131.

454. **Parolein** (a pure form of paraffinum liquidum) is recommended by Coles (*Lancet*, 1911, p. 878) as being quite neutral and preserving certain coal tar stains. Ring mounts with Apáthy's gum syrup, § 343. Its index is 1·471, which I find too low for most things.

455. **Cedar Oil.**—See § 442, *sub. fin.*

456. **Gum Thus**, dissolved in xylol, is recommended by Eisen, *Zeit. wiss. Mik.*, xiv, 1897, p. 201.

457. **Styrax and Liquidambar.**—See *Journ. Roy. Mic. Soc.*, 1883, p. 741; *ib.*, 1884, pp. 318, 475, 655, and 827; and the places there quoted. Also *Bull. Soc. Belge de Mic.*, 1884, p. 178; and Fol, *Lehrb.*, p. 141. These are very highly refractive media, therefore seldom useful in histology.

CHAPTER XX.

CEMENTS AND VARNISHES.

458. Introduction.—Two, or at most three, of the media given below will certainly be found sufficient for all useful purposes. For many years I have used only one cement (Bell's). I recommend this both as a cement and varnish; gold size may be found useful for turning cells; and Miller's caoutchouc cement may be kept for occasions on which the utmost solidity is required. Marine glue is only necessary for making glass cells.

For the operations of mounting in fluids, and of making cells and ringing, see Carpenter's *The Microscope.*

Carpenter lays great stress on the principle that the cements or varnishes used for fluid mounts should always be such as contain *no mixture of solid particles,* for those that do always become porous after a certain lapse of time. All fluid mounts *should have the edges of the cover carefully dried and be ringed with glycerin jelly before applying a cement; by this means all danger of running in is done away with.* See §§ 460 and 461. But no method yet devised will make a glycerine mount absolutely permanent.

See also Aubert, *The Microscope,* xi, 1891, 150, and *Journ. Roy. Mic. Soc.,* 1891, p. 692; Beck, *The Microscope,* xi, 1891, pp. 338, 368, and *Journ. Roy. Mic. Soc.,* 1892, p. 293; Behrens' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten* (Bruhn, Braunschweig, 1892); Rousselet, *Journ. Quek. Mic. Club,* vii, 1898, p. 93; and as to the comparative *tenacity* of divers cements, Behrens, *Zeit wiss. Mik.,* ii, 1885, p. 54, and Aubert *Amer. Mon. Mic. Journ.,* 1885, p. 227; *Journ. Roy. Mic. Soc.,* 1886, p. 173.—Behrens gives the palm to amber varnish; Aubert places Miller's caoutchouc cement at the head of the list, Lovett's cement coming halfway down, and zinc white cement at the bottom, with less than one quarter the tenacity of the caoutchouc cement.

459. Paraffin.—*Temporary* mounts may be closed with paraffin, or white wax, by applying it with a bent wire, as described § 471, and be made more or less permanent by varnishing.

460. Gelatin Cement.—Marsh's *Section-cutting*, 2nd ed., p. 104).—Take half an ounce of Nelson's opaque gelatin, soak well in water, melt in the usual way, stir in 3 drops of creasote. It is used warm.

When the ring of gelatin has become quite set and dry, it may be painted over with a solution of bichromate of potash made by dissolving 10 grains of the salt in an ounce of water. This should be done in daylight, in order to render the gelatin insoluble. The cover may then be finished with Bell's cement. This process is particularly adapted for glycerin mounts.

461. The Paper Cell Method.—By means of two punches I cut out rings of paper of about a millimetre in breadth, and of about a millimetre smaller in diameter than the cover-glass. *Moisten* the paper ring with mounting fluid, and centre it on the slide. Fill the cell thus formed with mounting fluid; arrange the object in it; put the cover on; fill the annular space between the paper and the margin of the cover with glycerin jelly (a turn-table may be useful for this); and as soon as the gelatin has set turn a ring of gold-size on it, and when that is quite dry, varnish with Bell's cement.

For greater safety, the gelatin may be treated with bichromate, according to Marsh's plan, last §.

462. Rousselet's Method for Aqueous Mounts (*op. cit.*, § 458).—Close the mount with a ring of a mixture of two parts of a solution of damar in benzol and one part gold-size. When dry, put on three or four thin coats of pure gold-size at intervals of twenty-four hours, and finish with a ring of Ward's brown cement.

463. Ward's Brown Cement is a shellac-alcohol solution made by E. Ward, Oxford Road, Manchester. Its best solvent is a mixture of wood-naphtha and alcohol. Rousselet considers it the best shellac varnish he has met with, better than Bell's.

464. Bell's **Cement.**—Composition unknown. May be obtained from the opticians, or from J. Bell & Co., chemists, 338, Oxford Street, London. This varnish sets quickly. The cover should be ringed with glycerin jelly before applying the varnish, especially with glycerin. It is soluble in ether or chloroform. It is not attacked by oil of cedar.

465. Miller's **Caoutchouc Cement.**—Composition unknown. May be obtained from the opticians. A very tenacious and quickly drying cement. It may be diluted by a mixture of equal parts of chloroform and strong alcohol (see Rousselet, *Journ. Quek. Club.*, v, ii, 1895, p. 8).

466. Clarke's **Spirit-proof Cement.**—Mr. Ch. Rousselet has highly recommended this to me. It may be procured from Mr. J. Bolton, 25, Balshall Heath Road, Birmingham.

Rousselet finds it the best he has tried for alcoholic liquids, but not perfectly proof against watery media.

467. **Asphalt Varnish** (*Bitume de Judée*).—Unquestionably one of the best of these media, either as a cement or a varnish, *provided it be procured of good quality*. It can be procured from the opticians.

468. **Brunswick Black.**—See *early editions*, or Beale, *How to Work*, etc., p. 49.

469. **Gold-Size.**—Best obtained from the opticians. It is soluble in oil of turpentine. A good cement, *when of good quality*, and very useful for turning cells.

470. **Marine Glue.**—Found in commerce. Carpenter says the best is that known as G K 4. Best obtained from the opticians. It is soluble in ether, naphtha, or solution of potash. Its use is for attaching glass cells to slides, and for

all cases in which it is desired to cement glass to glass. Used warm.

471. Turpentine, Venice Turpentine (Csokor, *Arch. mik. Anat.*, xxi, 1882, p. 353; Parker, *Amer. Mon. Mik. Journ.*, ii, 1881, pp. 229–30).—Venice turpentine, or common resinous turpentine, evaporated by heat until brittle on cooling. It is used for closing glycerin mounts in the following manner: Square covers are used, and superfluous glycerin is cleaned away from the edges in the usual way. The cement is then put on with a piece of wire bent at right angles; the short arm of the wire should be just the length of the side of the cover-glass. The wire is heated in a spirit lamp, plunged into the cement, some of which adheres to it, and then brought down flat upon the slide at the margin of the cover. The turpentine distributes itself evenly along the side of the cover, and hardens immediately, so that the slide may be cleaned as soon as the four sides are finished. It is claimed for this cement that it is perfectly secure and never runs in. It sets hard in a few seconds.

472. Colophonium and Wax (Krönig, *Arch. mik. Anat.*, 1886, p. 657).—Seven to 9 parts of colophonium are added piecemeal to 2 parts of melted wax, the whole filtered and left to cool. For use, the mass is melted by placing the containing vessel in hot water. The cement is not attacked by water, glycerin, or caustic potash.

Vosseler (*Zeit. wiss. Mik.*, vii, 1891, p. 462) takes 1 part of Venice turpentine to 2 to 3 of white wax.

473. Apáthy's **Cement for Glycerin Mounts** (*Zeit. wiss. Mik.*, vi, 1889, p. 171).—Equal parts of hard (60° C. melting-point) paraffin and Canada balsam. Heat together in a porcelain capsule until the mass takes on a golden tint and no longer emits vapours of turpentine. Used by warming and applying with a glass rod or brass spatula. One application is enough. Does not run in, and never cracks.

474. Canada Balsam, or Damar.—Cells are sometimes made with these. They are elegant, but in my experience are not reliable for permanent mounts.

475. Amber Varnish.—Behrens finds this cement to possess an extreme tenacity. That used by him may be obtained from Grübler & Hollborn.

476. **Amber and Copal Varnish** (HEYDENREICH, *Zeit. wiss. Mik.*, 1885, p. 338).—Extremely complicated.

477. **Shellac Varnish** (BEALE, p. 28).—A thick solution of shellac in alcohol. The *Micro. Dictionary* says that the addition of 20 drops of castor oil to the ounce is an improvement. Untrustworthy.

478. **Sealing-Wax Varnish** (*Micro. Dict.*, "Cements").—Add enough spirit of wine to cover coarsely powdered sealing-wax, and digest at a gentle heat. This should only be used as a varnish, never as a cement, as it is apt to become brittle and to lose its hold upon glass after a time.

479. **Tolu Balsam Cement** (CARNOY'S *Biol. Cell.*, p. 129).—Tolu balsam, 2 parts, Canada balsam 1, saturated solution of shellac in chloroform, 2 parts. Add enough chloroform to bring the mixture to a syrupy consistence. Carnoy finds this cement superior to all others.

PART II.

SPECIAL METHODS AND EXAMPLES.

CHAPTER XXI.

INJECTION—GELATIN MASSES (WARM).

480. Introduction.—Injection masses are composed of a coloured substance called the *colouring mass*, and of a substance with which that is combined called the *vehicle*.

For instructions as to the operation of injecting, and the necessary apparatus, see *the Micrographic Dictionary*, RUTHERFORD'S and SCHÄFER'S *Practical Histology*, the treatises of ROBIN and RANVIER, BEALE'S *How to Work with the Microscope*, the *Lehrbuch der vergleichenden Mikroscopischen Anatomie* of FOL, and (for apparatus especially) the article in the *Encycl. d. mik. Technik.* For injections for the study of the angiology of Vertebrates the practice of Robin and Ranvier may safely be followed. For injections of Invertebrates (and indeed, for vertebrates if it is desired to demonstrate the minute structure of environing tissues at the same time as the distribution of vessels) masses not containing gelatin are, I think, generally preferable to gelatin masses; and I would recommend as particularly convenient the Prussian blue glycerin masses of BEALE. Glycerin masses have the great advantage that they are used *cold.*

All formulæ which only give opaque masses, or are only suitable for coarse injections for naked eye study, have been suppressed.

481. Vaso-dilators.—In order that an injection may run freely it is necessary that the vessels of the subject be in a relaxed state. To this end the older anatomists used to wait until *rigor mortis* had passed off before injecting. But it is evidently preferable in the interest of the proper preservation of the tissues to inject before *rigor mortis* has set in. Unfortunately, when this is done, it is found that most injection-masses—glycerin masses especially—stimulate the contraction of the vessels, so that frequently it is very difficult to get the injection in. In these cases it may be advisable to use a vaso-dilator. The animal may be anæsthetised with a mixture of ether and *nitrite of amyl,* and finally killed with pure nitrite. Or, after killing by nitrite, a little nitrite of amyl in salt solution may be injected before the injection mass is thrown in. In any case it is advisable to add a little nitrite to the mass just before using. The relaxing power is very great (see OVIATT and SARGENT, in *St. Louis Med. Journ.,* 1886, p. 207; and *Journ. Roy. Mic. Soc.,* 1887, p. 341).

Or, *morphia* may be added to the injection mass, or one per cent. of *lactic acid.* MOZEJKO (*Zeit. wiss. mik.,* xvi, 1909, p. 545) prefers a saturated solution of *Peptonum siccum,* which has the advantage of hindering coagulation. For warm-blooded animals the mass should be warmed to body-temperature; and in all cases masses that tend to dehydrate tissues should be avoided if possible.

ROBIN'S *Masses.*

482. ROBIN'S **Gelatin Vehicle** (*Traité,* p. 30).—One part of gelatin soaked and melted in 7, 8, 9, or even 10 parts of water, on a water bath.

This vehicle, like all gelatin masses, is liable to be attacked by mould if kept long; camphor and carbolic acid do not suffice to preserve it. Chloral hydrate 2 per cent. is said to do so.

483. ROBIN'S **Glycerin-gelatin Vehicle** (*Traité,* p. 32).—Dissolve in a water-bath 50 grms. of gelatin in 300 grms. of water, in which has been dissolved some arsenious acid; add of glycerine 150 grms., and of carbolic acid a few drops. Unlike the pure gelatin vehicles, this mass does keep indefinitely.

FRANKL (*Zeit. f. wiss. Zool.,* lxiii, 1897, p. 28) prepares a similar vehicle, and adds to it a little solution of corrosive sublimate and a crystal of thymol.

484. ROBIN'S **Carmine Colouring Mass** (*Traité,* p. 33).—Rub up 3 grms. of carmine with a little water and enough ammonia to dissolve it. Add 50 grms. of glycerin and filter.

Take 50 grms. of glycerin with 5 grms. of acetic acid, and add it by degrees to the carmine-glycerine, until a slightly acid reaction is obtained (as tested by very sensitive blue test-paper, moistened and held over the mixture).

One part of this mixture is to be added to 3 or 4 parts of the vehicles given above.

485. Robin's Ferrocyanide of Copper Colouring Mass (*ibid.*, p. 34).—Take—

(1)	Ferrocyanide of potassium (concentrated solution)	20 c.c.
	Glycerin	50 ,,
(2)	Sulphate of copper (concentrated solution)	35 ,,
	Glycerin	50 ,,

Mix (1) and (2) slowly, with agitation; at the moment of injecting combine with 3 volumes of vehicle.

486. Robin's **Prussian Blue Colouring Mass** (*ibid.*, p. 35, and 2nd ed., p. 1013).

Take—

(A)	Ferrocyanide of potassium* (sol. sat.)	90 c.c.
	Glycerin	50 ,,
(B)	Liquid perchloride of iron at 30° Baumé	3 ,,
	Glycerin	50 ,,

Mix slowly and combine the mixture with 3 parts of vehicle. It is well to add a few drops of HCl.

Carmine-gelatin Masses.

487. Ranvier's **Carmine Gelatine Mass** (*Traité technique*, p. 116).—Take 5 grms. Paris gelatin, soak until quite swollen and soft, wash, drain and melt it in the water it has absorbed over a water bath. When melted add slowly, and with continual agitation, 2½ grms. of carmine rubbed up with a little water, and just enough ammonia, added drop by drop, to dissolve the carmine into a *transparent* solution.

The mixture is now neutralised by adding cautiously, drop by drop, with continual agitation, a solution of 1 part of glacial acetic acid in two parts of water. (When the mass is near neutrality, dilute the acetic acid still further.) The instant of saturation is determined by the smell of the solution, which gradually changes from ammoniacal to sour. As soon as the sour smell is perceived the liquid must be examined under the microscope. If it contains a granular precipitate of carmine, too much acid has been added, and it must be thrown away.

---

* *Erratum* "Sulphocyanide" in 1st edition of Robin's *Traité*.

The mass, having been perfectly neutralised, is strained through new flannel.

488. How to Neutralise a Carmine Mass (VILLE, *Gaz. hebd. d. Sci. méd. de. Montpellier*, Fév., 1882; may be had separately from Delahaye et Lecrosnier, Paris).—VILLE points out that when carmine is treated with ammonia a certain proportion of the ammonia combines with the carmine and the rest remains in excess. It is this *excess* that it is required to neutralise precisely, not the *whole* of the ammonia employed.

To neutralise the acidity of commercial gelatin, it should be washed for an hour or so in running water.

As to the neutralisation of the colouring mass, VILLE is of opinion that the sour smell cannot be safely relied on in practice, and prefers to employ dichroic litmus paper (litmus paper sensitised so as to be capable of being used equally for the demonstration of acids and bases). For directions for preparing this see *loc. cit.* or *previous editions.*

489. HOYER'S **Carmine-Gelatin Mass** (*Biol. Centralb.*, 1882, p. 21).—Take a concentrated gelatin solution and add to it the needful quantity of neutral carmine staining solution (*loc. cit.*, p. 17). Digest in a water-bath until the dark violet-red colour begins to pass into a bright red tint. Then add 5–10 per cent. by volumes of glycerin, and at least 2 per cent. by weight of chloral, in a concentrated solution, and strain.

490. FOL'S **Carmine-Gelatin Mass** (*Lehrb.*, p. 13). This can be *kept in the dry state* for an indefinite length of time.

Gelatin in sheets is cut into strips which are macerated for two days in carmine solution (prepared by diluting one volume of strong ammonia with three of water and adding carmine to *saturation*, and filtering after a day or two). The strips are then rinsed and put for a few hours into water acidulated with acetic acid, then washed on a sieve for several hours in running water, dried on parchment paper, or on a net, and preserved for future use. To get the mass ready for use, the strips are soaked for an hour in water, and melted on a water-bath in 10 to 20 parts of water.

For another process, which is said to give somewhat better results, but is more complicated, see *loc. cit.*, or *Zeit. wiss. Zool.*, xxxviii, p. 492, or *previous editions*.

491. KRAUSE's **Carmine-Gelatin Mass** (*Zeit. wiss. Mik.*, xxvi, 1909, p. 1).—100 grms. gelatin soaked in water, put for two to three days into a solution of 15 grms. carmine in 2 litres of water with 100 grms. of borax, washed, treated for a short time with hydrochloric acid of 2 per cent., washed, melted and preserved with camphor.

492. Other Carmine Gelatin Masses.—THIERSCH'S, see *Arch. mik. Anat.*, 1865, p. 148. GERLACH'S, see RANVIER, *Traité*, p. 118, CARTER'S, see BEALE, p. 113. DAVIES, see his *Prep. and Mounting of Mic. Objects*, p. 138.

Blue Gelatin Masses.

493. ROBIN'S **Prussian Blue Gelatin Mass** (see § 486).

494. RANVIER'S **Prussian Blue Gelatin Mass** (*Traité*, p. 119).—Make a concentrated solution of sulphate of peroxide of iron in distilled water, and pour it *gradually* into a concentrated solution of yellow prussiate of potash. There is produced a precipitate of insoluble Prussian blue. Wash this on a felt strainer, underneath which is arranged a paper filter in a glass funnel, for some days, until the liquid begins to run off blue from the second filter. The Prussian blue has now become soluble. The strainer is turned inside out and agitated in distilled water; the Prussian blue will dissolve if the quantity of water be sufficient.

The solution may now be injected just as it is, or it may be kept in bottles till wanted, or evaporated in a stove, and the solid residuum put away in bottle.

For injections, if a simple aqueous solution be taken, it should be *saturated*. Such a mass never transudes through the walls of vessels. Or it may be combined with one fourth of glycerin, or with one twenty-fifth of gelatin soaked for an hour in water and melted over a water bath in the water it has absorbed. The gelatin is to be poured gradually into the Prussian blue, on the water bath, stirring continually until the curdy precipitate that forms at first has disappeared. Filter through new flannel and keep at 40° C. until injected.

495. Brücke's Soluble Berlin Blue (*Arch. mik. Anat.*, 1865, p. 87).—Make a solution of ferrocyanide of potassium containing 217 grms. of the salt to 1 litre of water, and one of 1 part commercial chloride of iron in 10 parts water. Take equal volumes of each, and add to each of them twice its volume of a cold saturated solution of sulphate of soda. Pour the chloride solution into the ferrocyanide solution, stirring continually. Wash the precipitate on a filter until soluble, dry it, press between blotting paper in a press, break the mass in pieces, and dry in the air.

The concentrated solution of the colouring matter is to be gelatinised with just so much gelatin that the mass forms a jelly when cold. For another method, see *previous editions.*

496. Other Blue Gelatin Masses.—Hoyer's, *Arch. mik. Anat.*, 1876, p. 649; Guignet's, *Journ. de Microgr.*, 1889, p. 94; *Journ. Roy. Mic. Soc.*, 1889, p. 463; Thiersch's, *Arch. mik. Anat.*, i, 1865, p. 148; Fol's, *Zeit. wiss. Zool.*, xxxviii, 1883, p. 494; and *previous editions.*

Other Colours.

497. Hoyer's Silver Nitrate Yellow Gelatin Mass (*Biol. Centralbl.*, ii, 1882, pp. 19, 22).—A concentrated solution of gelatin is mixed with an equal volume of a 4 per cent. solution of nitrate of silver and warmed. To this is added a very small quantity of an aqueous solution of pyrogallic acid, which reduces the silver in a few seconds; chloral and glycerin are added as directed § 489.

This mass is yellow in the capillaries and brown in the larger vessels.

498. Other Colours.—Hoyer's **Green** (*Biol. Centralb.*, ii, 1882. p. 19). Made by mixing a blue mass and a yellow mass. Thiersch's **Green** (*Arch. mik. Anat.*, 1865, p. 149). Robin's Scheele's **Green** (Robin, *Traité*, p. 37). Harting's **White** (see Frey, *Le Microscope*, p. 190). Frey's **White** (*ibid.*). Teichmann's **White** (*ibid.*, p. 191). Fol's **Brown** (*Zeit. wiss. Zool.*, xxxviii, 1883, p. 494). Miller's **Purple** (see *Amer. Mon. Mic. Journ.*, 1888, p. 50; *Journ. Roy. Mic. Soc.*, 1888, p. 518). Fol's **Lead Chromate** (*Lehrb.*, p. 15). Robin's **Cadmium** (his *Traité*, p. 36). Thiersch's **Lead Chromate** (*Arch. mik. Anat.*, 1865, p. 149). Hoyer's **Lead Chromate** (*ibid.*, 1867, p. 136); or, for any of these, see *early editions.*

499. Ranvier's **Gelatin Mass for Impregnation** (*Traité*, p. 123).—Concentrated solution of gelatin, 2, 3, or 4 parts; 1 per cent. nitrate of silver solution, 1 part.

Neuville (*Ann. Sci. Nat.*, xiii, 1901, p. 36) takes a solution of 10 grms. of soaked gelatin in 100 c.c. of 1 per cent. solution of nitrate of silver.

500. Friedenthal's **Hardening Mass** (*Centralb. Phys.*, xiii, 1899, p. 267).—A 10 per cent. solution of gelatin, combined with a colouring mass, and with 1 vol. of 4 per cent. formol, serves for injecting vessels and hardening the tissues at the same time.

CHAPTER XXII.

INJECTIONS—OTHER MASSES (COLD).

501. Fol's **Metagelatin Vehicle** (*Lehrb.*, p. 17).—If a slight proportion of ammonia be added to a solution of gelatin, and the solution be heated for several hours, the solution passes into the state of metagelatin, that is, a state in which it no longer coagulates on cooling and can be injected without warming. Colouring masses may be added to this vehicle, which may also be thinned by the addition of weak alcohol. After injection the preparations are thrown into strong alcohol or chromic acid, which sets the mass.

According to the *Encycl. mik. Technik.*, metagelatin is usually prepared by warming with concentrated acetic or oxalic acid. It may be neutralised afterwards with carbonate of lime.

502. Tandler's **Gold Gelatin Mass** (*Zeit. wiss. mik.*, xviii, 1901, p. 22).—Five grms. of gelatin are soaked in 100 c.c. of water, warmed and melted, and combined with Berlin blue. Then 5 to 6 grms. of iodide of potassium are slowly incorporated. The mass generally remains liquid enough for injection down to a temperature of 17° C., but if it should coagulate a little more iodide should be added. After injection you may fix with 5 per cent. formol. The specimens will bear decalcification with hydrochloric or sulphurous acid.

Pearl (*Journ. Appl. Micr.*, v, 1902, p. 1736) takes 8 to 10 per cent. of the iodide.

Mayer (*Grundzüge* Lee and Mayer, 1910, p. 250) takes simply 10 grms. gelatin, 10 grms. *hydrate of chloral* and 100 c.c. water.

Mozejko (*Zeit. wiss. Mik.*, xxvii, 1910, p. 374), finds that

10 per cent (or more) of sodium salicylate will retard the setting of gelatin for hours at normal temperatures.

Any of these masses may be made to set in the tissues by means of weak formol.

Glycerin Masses.

503. **Beale's Carmine Glycerine Mass** (*How to Work*, etc., p. 95).—Five grains of carmine are dissolved in a little water with about five drops of ammonia, and added to half an ounce of glycerin. Then add half an ounce of glycerin with eight or ten drops of acetic or hydrochloric acid, gradually, with agitation. Test with blue litmus paper, and if necessary add more acid till the reaction is decidedly acid. Then add half an ounce of glycerin, two drachms of alcohol, and six drachms of water. I have found this useful, but not so good as the two following.

504. **Beale's Prussian Blue** (*How to Work*, etc., p. 93).

Common glycerin	1 ounce.
Spirits of wine	1 ,,
Ferrocyanide of potassium . . .	12 grains.
Tincture of perchloride of iron . .	1 drachm.
Water	4 ounces.

Dissolve the ferrocyanide in one ounce of the water and glycerin, and add the tincture of iron to another ounce. These solutions should be mixed together *very gradually*, and well shaken in a bottle, *the iron being added to the solution of the ferrocyanide of potassium.* Next the spirit and the rest of water are to be added very gradually, the mixture being constantly shaken.

Injected specimens should be preserved in acidulated glycerin (*e.g.* with 1 per cent. acetic acid), otherwise the colour may fade.

505. **Beale's Acid Prussian Blue** (*ibid.*, p. 296).

Price's glycerin	2 fluid ounces.
Tinct. of sesquichloride of iron . .	10 drops.
Ferrocyanide of potassium . . .	3 grains.
Strong hydrochloric acid . . .	3 drops.
Water	1 ounce.

Proceed as before, dissolving the ferrocyanide in one half of the glycerin, the iron in the other, and adding the latter drop by drop to the former. Finally add the water and HCl. Two drachms of alcohol may be added to the whole if desired.

I find this excellent.

506. **RANVIER's Prussian Blue Glycerin Mass** (*Traité*, p. 120).—The Prussian blue fluid, § 494, mixed with one fourth of glycerin.

507. **THOMA's Indigo-Carmine** (*Arch. Anat. Phys., Anat. Abth.*, 1899, p. 270).—Dissolve 0·15 grm. sulphindigotate of soda in 50 c.c. water, filter, add 40 c.c. glycerin and gradually, with agitation, 10 c.c. of a filtered 10 per cent. solution of sodium chloride in water. If desired, 3 c.c. of a 1 per cent. solution of morphia may be added to dilate arteries. A fine precipitate is formed, which is injected with the mass.

508. **Gamboge Glycerin** (HARTING, *Das Mikroskop*, 1866, 2, Theil, p. 124).—Gamboge rubbed up with water and added to glycerin; or a saturated alcoholic solution of gamboge added to a mixture of equal parts of glycerin and water. Any excess of alcohol may be got rid of by allowing the mass to stand for twenty-four hours.

509. **Other Colours.**—Any of the colouring masses, §§ 485 to 498, or other suitable colouring masses, combined with glycerin, either dilute or pure.

Purely Aqueous Masses.

510. **RANVIER's Prussian Blue Aqueous Mass** (*Traité*, p. 120). —The soluble Prussian blue, § 494, injected without any vehicle. It does not extravasate.

511. **MÜLLER's Berlin Blue** (*Arch. Mik. Anat.*, 1865, p. 150).—Precipitate a concentrated solution of Berlin blue by means of ½ to 1 volume of 90 per cent. alcohol. The precipitate is very finely divided; and the fluid may be injected at once.

512. **MAYER's Berlin Blue** (*Mitth. Zool. Stat. Neapel*, 1888, p. 307.)—A solution of 10 c.c. of tincture of perchloride of

iron in 500 c.c. of water is added to a solution of 20 gr. of yellow prussiate of potash in 500 c.c. of water, allowed to stand for twelve hours, decanted, the deposit washed with distilled water on a filter until the washings come through dark blue (one to two days), and the blue dissolved in about a litre of water. It is well to add a little acetic acid and to put up the objects in an acid liquid.

513. EMERY'S **Aqueous Carmine** (*ibid.*, 1881, p. 21).—To a 10 per cent. ammoniacal solution of carmine is added acetic acid, with continual stirring, until the colour of the solution changes to blood-red. The supernatant clear solution is injected cold without further preparation. The injected organs are thrown at once into strong alcohol to fix the carmine. For injection of fishes.

514.—TAGUCHI'S **Indian Ink** (*Arch. mik. Anat.*, 1888, p. 565). —Chinese or (better) Japanese ink well rubbed up on a hone until a fluid is obtained that does not run when dropped on thin blotting-paper, nor form a grey ring round the drop. Inject until the preparation appears quite black, and throw it into some hardening liquid (not pure water).

DELLA ROSA (*Ver. Anat. Ges.*, 1900, p. 141) recommends the liquid Chinese ink sold in the shops.

Partially Aqueous Masses.

515. JOSEPH'S **White-of-Egg** (*Ber. naturw. Sect. Schles. Ges.*, 1879, pp. 36—40; *Journ. Roy. Mic. Soc.*, ii, 1882, p. 274).—"Filtered white-of-egg, diluted with 1 to 5 per cent. of carmine solution. . . . This mass remains liquid when cold, coagulates in dilute nitric acid, chromic or osmic acid, and remains transparent in the vessels." For invertebrates.

GROSSER (*Zeit. wiss. Mik.*, xvii, 1900, p. 178) rubs up Indian ink with white-of-egg; HOFFMANN (*Zeit. Morph. Anthrop.*, iii, 1901, p. 240) with blood-serum; so also HAMBURGER, *Zeit. wiss. Mik.*, xxv, 1908, p. 1 (2 vols. of the ink—"Perltusche"—to 3 of serum).

516. BJELOUSSOW'S **Gum Arabic Mass** (*Arch. Anat. Phys.*, 1885, p. 379).—Make a syrupy solution of gum arabic and a saturated solution of borax in water. Mix the solutions in such proportions as to have in the mixture 1 part of borax

to 2 of gum arabic. Rub up the transparent, almost insoluble mass with distilled water, added little by little, then force it through a fine-grained cloth. Repeat these operations until there is obtained a mass that is free from clots. It should then coagulate in the presence of alcohol, undergoing at the same time a dilatation to twice its original volume. The vehicle thus prepared may be combined with any colouring mass except cadmium and cobalt.

After injection the preparation is thrown into alcohol, and the mass sets immediately, swelling up as above described, and consequently showing vessels largely distended.

Cold-blooded animals may be injected whilst alive with this mass. It does not flow out of cut vessels. Injections keep well in alcohol. If it be desired to remove the mass from any part of a preparation, this is easily done with dilute acetic acid.

517. **Milk** has been recently recommended by FISCHER (*Centralb. allg. Path.*, xiii, 1902, p. 277; *Zeit. wiss. Mik.* xx, 1903, p. 224). It runs well, does not extravasate, and can be used for auto-injection of the living subject.

After injection it should be coagulated by putting the organs for at least twenty-four hours into a mixture of 75 parts of formol, 15 of acetic acid, and 1000 of water (pure formol will not do). They are then sectioned, and the sections stained with Sudan III or Scharlach R, which stain the milk. They cannot be mounted in balsam.

Celloidin and other Masses.

518. SCHIEFFERDECKER'S **Celloidin Masses** (*Arch. Anat. Phys.*, 1882 [*Anat. Abth.*], p. 201). (For Corrosion preparations).—See *previous editions*; HOCHSTETTER'S **Modification of** SCHIEFFERDECKER'S **Mass** (*Anat. Anz.*, 1886, p. 51); BUDGE'S **Asphaltum Mass** (*Arch. Mik. Anat.*, xiv, 1877, p. 70), or *early editions*; HOYER'S **Shellac Mass** (*Arch. Mik. Anat.*, 1876, p. 645). For this and that of BELLARMINOW (*Anat. Anz.*, 1888, p. 605), see *early editions*; HOYER'S **Oil-colour Masses** (*Internat. Monatsschr. Anat.*, 1887, p. 341); SEVEREANU'S, *Verh. Anat. Ges.*, 21 *vers*, 1906, p. 275; PANSCH'S **Starch Mass** (*Arch. Anat. Entw.*, 1877, p. 480; 1880, pp. 232, 371; 1881, p. 76; 1882, p. 60; 1883, p. 265; and a modification of the same by GAGE, *Amer. Mon. Mic. Journ.*, 1888, p. 195); TEICHMANN'S **Linseed-Oil Masses** (*S. B. Math. Kl. Krakau Akad.*, vii, pp. 108, 158; *Journ. Roy. Mic. Soc.*, 1882, pp. 125 and 716, and 1895, p. 704).

Flint's **Celluloid** (*Amer. Journ. Anat.*, i, 1902, p. 270); Huber's (*ibid.*, vi, 1907, p. 393); Krassuskaja's **Photoxylin** (*Anat.*, Heft. 2, xiii, 1904, p. 521).

519. Natural Injections (Robin, *Traité*, p. 6).—To preserve these throw the organs into a liquid composed of 10 parts of tincture of perchloride of iron and 100 parts of water.

Retterer and Zenker use solution of Müller, see *Journ. Anat. Phys.*, 1894, p. 336, and *Arch. Path. Anat.*, 1894, p. 147.

CHAPTER XXIII.

MACERATION, DIGESTION, AND CORROSION.

Maceration.

520. Methods of Dissociation.—It is sometimes necessary, in order to obtain a complete knowledge of the forms of the elements of a tissue, that the elements be artificially separated from their place in the tissue and separately studied after they have been isolated both from neighbouring elements and from any interstitial cement-substances that may be present in the tissue. Simple teasing with needles is often insufficient, as the cement-substances are frequently tougher than the elements themselves, so that the latter are torn and destroyed in the process. In this case recourse must be had to maceration, by which is meant prolonged soaking (generally for days rather than hours) in media which have the property of dissolving, or at least softening, the cement substances or the elements of the tissue that it is not wished to study, whilst preserving the forms of those it is desired to isolate. When this softening has been effected, the isolation is completed by teasing, or by agitation with liquid in a test-tube, or by the method of tapping, which last gives in many cases (many epithelia, for instance) results which could not be attained in any other way. The macerated tissue is placed on a slide and covered with a thin glass cover supported at the corners on four little feet made of pellets of soft wax. By tapping the cover with a needle it is now gradually pressed down, whilst at the same time the cells of the tissue are segregated by the repeated shocks. When the segregation has proceeded far enough, mounting medium may be added and the mount closed.

A good material for making *wax feet* is obtained (VOSSELER, *Zeit. wiss. Mik.*, vii, 1891, p. 461) by melting white wax and stirring into it one half to two thirds of Venice turpentine.

The most desirable macerating media are those which, whilst dissolving intercellular substances, do not attack the cells themselves. Those which contain *colloids* have been found to give the best results in this respect. Iodised serum is an example.

521. **Iodised Serum** (Chap. XIX).—The manner of employing it for maceration is as follows: A piece of tissue smaller than a pea must be taken, and placed in 4 or 5 c.c. of weakly iodised serum in a well-closed vessel. After one day's soaking the maceration is generally sufficient, and the preparation may be completed by teasing or pressing out, as indicated last §; if not, the soaking must be continued, fresh iodine being added as often as the serum becomes pale by the absorption of the iodine by the tissues. By taking this precaution the maceration may be prolonged for several weeks.

This method is intended to be applied to the preparation of *fresh* tissues, the iodine playing the part of a fixing agent with regard to protoplasm, which it slightly hardens.

522. **Iodide of Potassium** (ARNOLD, *Arch. mik. Anat.*, lii, 1898, pp. 135 and 763).—10 c.c. of 10 per cent. aqueous sol. of potassic iodide with 5 to 10 drops of a similar solution, containing also 5 per cent. of iodine.

523. **Alcohol.**—RANVIER employs one-third alcohol (1 part of 90 per cent. alcohol to 2 parts of water). Epithelia will macerate well in this in twenty-four hours. It macerates more rapidly than iodised serum.

Other strengths of alcohol may be used, either stronger (equal parts of alcohol and water) or weaker (¼ alcohol, for isolation of the nerve-fibres of the retina, for instance—THIN).

524. **Salt Solution.**—10 per cent. solution of sodium chloride is a valuable macerating medium. Weaker strengths, down to 0·6 per cent., are also used.

525. MOLESCHOTT and PISO BORME'S **Sodium Chloride and Alcohol** (MOLESCHOTT'S *Untersuchungen zur Naturlehre*, xi, pp. 99—107; RANVIER, *Traité*, p. 242).—10 per cent. solution of sodium chloride, 5 volumes; absolute alcohol, 1 volume.

For vibratile epithelium Ranvier finds the mixture inferior to one-third alcohol.

526. Sodium Chloride and Formaldehyde.—Gage recommends the addition of 2 parts of formalin to 1000 parts of normal salt solution (quoted from Fish, *Proc. Amer. Mic. Soc.*, xvii, 1895, p. 328).

527. Caustic Potash, Caustic Soda.—These solutions should be employed *strong*, 35 to 50 per cent. (Moleschott); so employed they do not greatly alter the forms of cells, whilst weak solutions destroy all the elements. (Weak solutions may, however, be employed for dissociating the cells of epidermis, hairs, and nails.) The strong solutions may be employed by simply treating the tissues with them on the slide. To make permanent preparations, the alkali should be neutralised by adding acetic acid, which forms with caustic potash acetate of potash, which constitutes a mounting medium (see Behrens, Kossel, and Schiefferdecker, *Das Mikroskop*, i, 1889, p. 156). See also Gage, *Proc. Amer. Soc. of Microscopists*, 1889, p. 35.

528. Baryta-water, Lime-water (Fol, *Lehrb.*, p. 110).—Baryta-water will macerate nerve, muscle, and connective tissue in a few hours, lime-water in a few days.

529. Sulphocyanides of Ammonium and Potassium (Stirling, *Journ. Anat. and Phys.*, xvii, 1883, p. 208).—10 per cent. solution of either of these salts, for epithelium. Macerate small pieces for twenty-four to forty-eight hours.

Soulier (*Travaux de l'Inst. Zool. de Montpellier*, Nouv. Sér., 2, 1891, p. 171) has found that Stirling's solution greatly deteriorates cellular elements, but that good results are obtained by *combining it with a fixing agent.* The best results were obtained with a 2 per cent. solution of sulphocyanide combined with liquid of Ripart and Petit; good ones, by combining liquid of Ripart and Petit with artificial serum of Kronecker instead of sulphocyanide, or with pepsin, eau de Javelle, 10 per cent. sulphate of soda, or 1·5 per cent. solution of caustic soda; also by combining solutions of chloride of sodium, or solutions of caustic potash or soda, with any of the usual fixing agents.

530. Landois's Solution (*Arch. mikr. Anat.*, 1885, p. 445).

Saturated sol. of neutral chromate of ammonia	5 parts.
Saturated sol. of phosphate of potash . .	5 „
Saturated sol. of sulphate of soda . . .	5 „
Distilled water	100 „

Small pieces of tissue are macerated for one to three, or even four to five days, in the liquid, then brought for twenty-four hours into ammonia carmine diluted with one volume of the macerating liquid.

Gierke particularly recommends this liquid for all sorts of macerations, but especially for the central nervous system, for which he finds it superior to all other agents. It is also recommended for the same purpose by Nansen (v. *Zeit. wiss. Mik.*, v, 1888, p. 242).

531. Bichromate of Potash.—0·2 per cent.

Eisig (*Fauna u. Flora Golf. Neapel*, 16 Monog., 1887, p. 297) macerates Capitellidæ in 0·5 to 1 per cent. solution for months or years, a little thymol being added against mould.

Müller's Solution, diluted to same strength, or combined with saliva, has also been used.

Brock (for nervous system of Mollusca, *Intern. Monatssch. Anat.*, i, 1884, p. 349) takes equal parts of 10 per cent. solution of bichromate of potash and visceral fluid of the animal.

532. Permanganate of Potash. — Is recommended, either alone or combined with alum, as the best dissociating agent for the fibres of the cornea (Rollett, *Stricker's Handbuch*, p. 1108). I have found it, for some objects, very energetic.

533. Chromic Acid.—Generally employed of a strength of about 0·02 per cent. Specially useful for nerve tissues and smooth muscle. Twenty-four hours' maceration will suffice for nerve tissue. About 10 c.c. of the solution should be taken for a cube of 5 mm. of the tissue (Ranvier).

534. Osmic and Acetic Acid (the Hertwigs, *Das Nervensystem u. die Sinnesorgane der Medusen*, Leipzig, 1878, and *Jen. Zeitschr.*, xiii, 1879, p. 457).

0·05 per cent. osmic acid . . 1 part.
0·2 „ acetic acid . . 1 „

Medusæ are to be treated with this mixture for two or three minutes, according to size, and then washed in repeated changes of 0·1 per cent. acetic acid until all traces of free osmic acid are removed; they then remain for a day in 0·1 per cent. acetic acid, are washed in water, stained in BEALE's carmine, and preserved in glycerin.

For *Actiniæ* the osmic acid is taken weaker, 0·04 per cent.; both the solutions are made with sea water; and the washing out is done with 0·2 per cent. acetic acid. If the maceration is complete, stain with picro-carmine; if not, with BEALE's carmine.

535. MÖBIUS's Media (*Morph. Jahrb.*, xii, 1887, p. 174).

1. One part of sea water with 4 to 6 parts of 0·4 per cent. solution of bichromate of potash.

2. 0·25 per cent. chromic acid, 0·1 per cent. osmic acid, 0·1 per cent. acetic acid, dissolved in sea water. For Lamellibranchiata. Macerate for several days.

536. Nitric Acid.—Most useful for the maceration of muscle. The strength used is 20 per cent. After twenty-four hours' maceration in this, isolated muscle-fibres may generally be obtained by shaking the tissue with water in a test-tube. Preparations may afterwards be washed with water and put up in strong solution of alum, in which they may be preserved for a long time (HOPKINS, *Proc. Amer. Soc. of Microscopists*, 1890, p. 165).

Maceration is greatly aided by heat, and at a temperature of 40° to 50° C. may be sufficiently complete in an hour (GAGE).

A mixture of equal parts of nitric acid, glycerin, and water is recommended by MARCACCI (*Arch. Ital. Biol.*, iv, 1883, p. 293) for smooth muscle.

537. Nitric Acid and Chlorate of Potash (KÜHNE, *Ueber die peripherischen Endorgane*, etc., 1862; RANVIER, *Traité*, p. 79).—Chlorate of potash is mixed, in a watch-glass, with four times its volume of nitric acid. A piece of muscle is buried in the mixture for half an hour, and then agitated with water in a test-tube, by which means it entirely breaks up into isolated fibres.

538. Nitric and Acetic Acid (APÁTHY, *Zeit. wiss. Mik.*, x, 1898, p. 49).—3 vols. glacial acetic acid, 3 of nitric acid, and 20 each of water, glycerin, and absolute alcohol. Macerate leeches for twenty-four hours, and bring them into 70 per cent. alcohol, in which they swell; then after twenty-four hours, 50 per cent. glycerin, changed till the acid is removed.

539. **Hydrochloric Acid.**—KÖNIGSTEIN (*Sitzb. Akad. Wien*, lxxi, 1875) takes (for gold-impregnated corneæ) equal parts of the concentrated acid, glycerin, and water; FREUD (*ibid.*, lxxviii, 1879, p. 102, for nerve-impregnations) 10 parts of acid, 7 of water, 3 of glycerin; and SCHUBERG and SCHRÖDER (*Zeit. wiss. Zool.*, lxxvi, 1904, p. 516) take (for fresh muscles of Hirudinea) hydrochloric acid of 5 per cent.

540. BÉLA HALLER'S **Mixture** (*Morphol. Jahrb.*, xi, p. 321). —One part glacial acetic acid, 1 part glycerin, 2 parts water. For the central nervous system of Mollusca a maceration of thirty to forty minutes may be sufficient.

541. **Sulphuric Acid** (RANVIER, *Traité*, p. 78).—Macerate for twenty-four hours in 30 grms. of water, to which are added 4 to 5 drops of concentrated sulphuric acid. Agitate. For nasal mucosa, crystalline, retina, etc.

ODENIUS found very dilute sulphuric acid to be the best reagent for the study of nerve endings in tactile hairs. He macerated hair-follicles for from eight to fourteen days in a solution of from 3 to 4 grains of "English sulphuric acid" to the ounce of water

Hot concentrated sulphuric acid serves to dissociate horny epidermic structures (horn, hair, nails).

542. **Oxalic Acid.**—Maceration for many days in concentrated solution of oxalic acid has been found useful in the study of nerve-endings.

543. SCHIEFFERDECKER'S **Methyl Mixture** (for the retina) (*Arch. mik. Anat.*, xxviii, 1886, p. 305).—Ten parts of glycerin, 1 part of methyl alcohol, and 20 parts of distilled water. Macerate for several days (perfectly fresh tissue).

544. GAGE'S **Picric Alcohol** (*Proc. Amer. Soc. of Microscopists*, 1890, p. 120).—95 per cent. alcohol, 250 parts; water, 750; picric acid, 1. Recommended especially for epithelia and muscle. A few hours suffice.

545. **Chloral Hydrate.**—In not too strong solution, from 2 to 5 per cent. for instance, chloral hydrate is a mild macerating

agent that admirably preserves delicate elements. Lavdowsky (*Arch. mik. Anat.*, 1876, p. 359) recommends it greatly for salivary glands, Hickson (*Quart. Journ. Mic. Sci.*, 1885, p. 244) for the retina of Arthropods.

546. **Lysol** (Reinke, *Anat. Anz.*, viii, 1892, p. 582).—10 per cent. solution in distilled water or in water with alcohol and glycerin. Spermatozoa of the rat or cortical cells of hairs are said to be resolved into fibrils in a few minutes, epithelial cells of salamandra to be dissociated instantaneously.

Digestion.

547. **Digestion** is maceration in organic juices, which by dissolving out some of the constituents of tissues earlier than others serves to isolate those which resist. The chief liquids employed are gastric juice (or pepsin) and pancreatic juice (pancreatin or trypsin).

Pepsin is best employed in acidified solution, pancreatin in alkaline.

The most favourable temperature for digestion is about 40° C.

Pepsin digests albuminoids, collagen substance and mucin, more or less readily, elastin more slowly. Nuclein is either not dissolved or very slowly. Keratin, neurokeratin, chitin, fat and carbohydrates are not attacked.

Pancreatin (trypsin) digests albuminoids, nuclein, mucin, and elastic tissue; whilst collagen substance, reticular tissue, chitin, horny substances, fat and carbohydrates are not attacked.

Tissues for digestion should be fresh, or fixed with alcohol, not with chromic acid or other salts of the heavy metals.

548. **Pepsin** (Beale's, *Archives of Medicine*, i, 1858, pp. 296—316).—The mucus expressed from the stomach glands of the pig is rapidly dried on glass plates, powdered, and kept in stoppered bottles. Eight tenths of a grain will dissolve 100 grains of coagulated white of egg.

To prepare the digestion fluid, the powder is dissolved in distilled water, and the solution filtered. Or the powder may be dissolved in glycerin. The tissues to be digested may be kept for some hours in the liquid at a temperature of 100° F. (37° C.)

Brücke's (from Carnoy's *Biologie cellulaire*, p. 94).

Glycerinated extract of pig's stomach . 1 vol.
0·2 per cent. solution of HCl . . . 3 vols.
Thymol, a few crystals.

Bickfalvi's (*Centralbl. med. Wiss.*, 1883, p. 838).—One grm. of dried stomachal mucosa is mixed with 20 c.c of 0·5 per cent. hydrochloric acid, and put into an incubator for three or four hours, then filtered. Macerate for not more than half an hour to an hour.

Kuskow's (*Arch. mik. Anat.*, xxx, p. 32).—One part of pepsin dissolved in 200 parts of 3 per cent. solution of oxalic acid. The solution should be freshly prepared, and the objects (sections of hardened Ligamentum Nuchæ) remain in it at the ordinary temperature for ten to forty minutes.

549. **Pancreatin.**—Schiefferdecker's (*Zeit. wiss. Mik.*, iii, 1886, p. 483).—A saturated solution of the "Pankreatinum siccum," prepared by Dr. Witte, Rostock, is made in distilled water, cold, and filtered. Pieces of tissue (epidermis) are macerated in it for three to four hours at about body temperature.

Kühne's (*Unters. a. d. Phys. Inst. Univ. Heidelberg*, i, 2, 1877. p. 219).—Very complicated.

See also Gedoelst, *La Cellule*, iii, 1887, p. 117, and v, 1889, p. 126; Maas, *Festschr. Kupffer*, 1899, p. 211, and Hoehl, *Arch. Anat. Phys.*, *Anat. Abth.*, 1897, p. 136 ($\frac{1}{5}$ to $\frac{2}{5}$ per cent. solution of Mall's or Merck's pancreatin, with 0·3 per cent. of carbonate of soda; for demonstrating adenoid tissue in paraffin sections).

Corrosion.

550. **Corrosion** is the operation of destroying the soft parts that surround hard parts that it is desired to study; in short, a means of cleansing hard parts for microscopic study. It has been applied to the removal of surrounding tissue from injected vessels or cavities. For this, see Altmann's Method (*Arch. mik. Anat.*, 1879, p. 471, or *previous editions*); also Rejsek (*Bibliogr. Anat.*, iv, 1897, p. 229); Brühl (*Anta. Anz.*, xiv, 1898, p. 418); Denker (*Anat. Hefte.*, 1900, p 300); Thoma and Fromherz (*Arch. Entwickelungsmech*, vii, 1898.

p. 678); Peabody (*Z. Bull.*, Boston, 1897, p. 164). The following sections relate chiefly to the cleansing of native hard parts.

551. Caustic Potash, Caustic Soda, Nitric Acid.—Boiling, or long soaking in a strong solution of either of these is an efficient means of removing soft parts from skeletal structures (appendages of Arthropods, spicula of sponges, etc.).

552. Eau de Javelle (Hypochlorite of Potash) (Noll, *Zool. Anzeig.*, 122, 1882, p. 528).—A piece of sponge, or similar object, is brought on to a slide and treated with a few drops of eau de Javelle, in which it remains until all soft parts are dissolved. (With thin pieces this happens in twenty to thirty minutes.) The preparation is then cautiously treated with acetic acid, which removes all precipitates that may have formed, dehydrated, and mounted in balsam.

The process is applicable to calcareous structures.

553. Eau de Labarraque (Hypochlorite of Soda) may be used in the same way as eau de Javelle. Looss (*Zool. Anzeig.*, 1885, p. 333) finds that either of these solutions will completely dissolve chitin in a short time with the aid of heat. For this purpose the commercial solution should be taken concentrated and boiling.

If solutions diluted with 4 to 6 volumes of water be taken, and chitinous structures be macerated in them for twenty-four hours or more, according to size, the chitin is not dissolved, but becomes transparent, soft and permeable to staining fluids, aqueous as well as alcoholic. The most delicate structures, such as nerve-endings, are stated not to be injured by the treatment. The method is applicable to Nematodes and their ova, and also to the removal of the albumen from ova of Amphibia, etc.

CHAPTER XXIV.

DECALCIFICATION, DESILICIFICATION, AND BLEACHING.

Decalcification.

554. Decalcification.—In order to obtain the best results, it is important to employ only material that has been *duly fixed and hardened*, and it is well not to put too much confidence in reagents that are said to have the property of hardening and decalcifying fresh material at the same time.

It is generally well also to employ fluids that contain substances having a shrinking action on tissues, so as to neutralise the swelling frequently brought about by the decalcifying acids. Large quantities of liquid should be employed.

After decalcification the excess of acid should be carefully removed by washing, not in water, which favours swelling, but in some liquid that has rather a shrinking action, *e. g.* alum solution. Lastly, the tissues should be neutralised by treatment with carbonate of lime, or a salt of lithium or sodium or the like.

Rousseau (*Zeit. wiss. Mik.*, xiv, 1897, p. 207) imbeds fixed material in celloidin, brings it into 85 per cent. alcohol, decalcifies in a very acid mixture (15 to 40 per cent. of nitric acid in alcohol), washes out the acid in alcohol containing precipitated carbonate of lime, then cuts sections. This for Porifera, corals, Echinoderms, etc. Tissues are said to be well preserved.

This process has been applied to the study of the temporal bone of Mammals by Stein (*Anat. Anz.*, xvii, 1900, p. 318).

Similarly Bödecker (*Zeit. wiss. Mik.*, xii, p. 190; xxv, p. 21; xxvi, p. 206; and xxviii, p. 158), in a complicated way, *adding* the acid (6 to 10 per cent.) to the *thin* celloidin solution taken for imbedding.

555. Decalcification of Bone.—I take the following from Busch, *Arch. mik. Anat.*, xiv, 1877, p. 481; see also Haug, in *Zeit. wiss. Mik.*, viii, 1891, p. 1; and Schaffer, *ibid.*, xix, 1903, pp. 308 and 441, and his paper in the *Encycl. mik. Technik.*

The most widely used, though not the best, agent for decalcification is *hydrochloric* acid. Its action is rapid, even when every dilute, but causes serious swelling of the tissues. To remedy this, chromic acid or alcohol may be added to it. Or a 3 per cent. solution of the acid may be taken and have dissolved in it 10 to 15 per cent. of common salt. Or (Waldeyer) to a $\frac{1}{1000}$ per cent. solution of *chloride of palladium* may be added $\frac{1}{10}$ of its volume of HCl.

Chromic acid is also much used, but has a very weak decalcifying action and a strong shrinking action on tissues. For this reason it should never be used in solutions of more than 1 per cent. strength, and for delicate structures much lower strengths must be taken.

Phosphoric acid has been recommended for young bones.

Acetic, lactic and *pyroligneous acids* have considerable decalcifying power, but cause great swelling. *Picric acid* has a very slow action, and is only suitable for very small structures.

556. Nitric Acid (Busch, *loc. cit.*).—To all other agents Busch prefers nitric acid, which causes no swelling and acts most efficaciously.

One volume of chemically pure nitric acid of sp. gr. 1·25 is diluted with 10 vols. water. It may be used of this strength for very large and tough bones; for young bones it may be diluted down to 1 per cent.

Fresh bones are first laid for three days in 95 per cent. alcohol; they are then placed in the nitric acid, *which is changed daily*, for eight or ten days. They must be *removed as soon as* the decalcification is complete, or else they will become stained yellow. When removed they are washed for one or two hours in running water and placed in 95 per cent. alcohol. This is changed after a few days for fresh alcohol.

Young and fœtal bones may be placed in the first instance in a mixture containing 1 per cent. bichromate of potash and $\frac{1}{10}$ per cent. chromic acid, and decalcified with nitric acid of 1 to 2 per cent., to which may be added a small quantity of chromic acid ($\frac{1}{10}$ per cent.) or bichromate of potash (1 per cent.). By putting them afterwards into alcohol a green stain is obtained.

557. **Nitric Acid** (SCHAFFER, *Zeit. wiss. Mik.*, xix, 1903, p. 460).—SCHAFFER also finds nitric acid the best reagent. It should be taken pure; the addition of formol, alcohol, or the like, slows the reaction. The best strength is from 3 to 5 per cent. Objects must not be washed out directly with water, and washing in salt solution, alcohol, phloroglucin, or formol is not sufficient to prevent swelling. Alum in 5 per cent. solution is good, but not necessary. Material should be well fixed and imbedded in celloidin (§ 554); harden in alcohol; remove the alcohol with water; put for 12 to 24 hours (large specimens longer) into nitric acid of 3 to 5 per cent, then into a 5 per cent. solution of sulphate of lithium or sodium, to be changed once in the course of 12 to 24 hours; running water, 48 hours; alcohol.

558. **Nitric Acid and Alcohol.**—3 per cent. of nitric acid in 70 per cent. alcohol. MAYER has long used 5 per cent. acid in 90 per cent. alcohol. Soak specimens for several days or weeks. Pure nitric acid, even if weak, readily exercises a gelatinising action on bone; whilst the addition of alcohol (or of *alum*) counteracts this action (FISH, *Ref. Handb. Med. Sci.*, Supp., p. 425).

THOMA (*Zeit. wiss. Mik.*, viii, 2, 1891, p. 191) takes five vols. of 95 per cent. alcohol and 1 volume pure concentrated nitric acid. Leave bones in this mixture, changing the liquid every two or three days, until thoroughly decalcified, which should happen, even with large bones, in two or three weeks. Wash out until every trace of acid is removed (*i. e.* for some days after no acid reaction is obtained with litmus paper) in 95 per cent. alcohol containing an excess of precipitated carbonate of lime. This may take eight to fourteen days, after which the tissues will stain well and may be treated as desired.

559. Nitric Acid and Formol.—Schridde (*Hæmatol. Techn.*, Jena, 1910, p. 21) decalcifies material fixed in formol or formol-Müller in a mixture of 1 part of formol, 1 of nitric acid, and 9 of water.

560. Nitric Acid and Alum (Gage, quoted from Fish, § 558).—A saturated aqueous solution of alum is diluted with an equal volume of water, and to each 100 c.c. of the dilute solution is added 5 c.c. of strong nitric acid. Change every two or three days, until the decalcification is complete. For teeth this is said to be, perhaps, a better decalcifier than the alcohol mixture.

561. Sulphurous Acid (Ziegler, *Festschr. f. Kupffer*, 1899, p. 51).—A saturated solution in water. Wash out for 24 hours. Acts rapidly and preserves well. Best used after fixation with formol.

562. Hydrochloric Acid (see § 555).—Ranvier says that it may be taken of 50 per cent. strength, and then has a very rapid action. To counteract the swelling action of the acid, sodium chloride may be added (von Ebner), see Haug's paper quoted § 555. He takes either 100 c.c. cold saturated solution of sodium chloride in water, 100 c.c. water, and 4 c.c. hydrochloric acid. Preparations to be placed in this, and 1 to 2 c.c. hydrochloric acid added daily until they are soft. Or, 2·5 parts of hydrochloric acid, 500 of alcohol, 100 of water, and 2·5 of sodium chloride. Haug prefers the proportions of 1·0 to 5·0 of acid, 70 of alcohol, 30 of water, and 0·5 of salt.

563. Hydrochloric Acid and Chromic Acid (Bayerl, *Arch. mik. Anat.*, 1885, p. 35).—Equal parts of 3 per cent. chromic acid and 1 per cent. hydrochloric acid. For ossifying cartilage. Haug recommends equal parts of 1 per cent. hydrochloric acid and 1 per cent. chromic acid (*loc. cit.*).

564. Hydrochloric Acid and Glycerin.—Glycerin, 95; hydrochloric acid, 5 (Squire's *Methods and Formulæ*, p. 12).

565. Trichloracetic Acid.—Partsch (*Verh. Ges. D. Naturf. Aertze*, 1895, 2 *Theil*, 2 Hälfte, p. 26) uses a 5 per cent. aqueous solution; and Neuberger (*Centralb. Phys.*, xi, 1897, p. 494) a 4 per cent. one. Action energetic, preservation said to be excellent.

566. **Picric Acid** should be taken saturated and changed frequently. Its action is weak, but it gives good results with small objects.

Picro-nitric or Picro-hydrochloric Acid.—Action very rapid.

567. **Phosphoric Acid.**—10 to 15 per cent. (HAUG, *loc. cit.* in § 555). Somewhat slow, staining not good. According to SCHAFFER, § 557, it produces swelling.

568. **Lactic Acid.**—10 per cent. or more. Fairly rapid, preserves well, and may be recommended (HAUG, *loc. cit.*).

569. **Chromic Acid** is employed in strengths of from 0·1 per cent. to 2 per cent. (but see § 555), the maceration lasting two or three weeks (in the case of bone). It is better to take the acid weak at first, and increase the strength gradually. Action excessively slow.

570. **Chromic and Nitric Acid.**—SEILER (FOL, *Lehrb.*, p. 112) takes 70 volumes of 1 per cent. chromic acid, 3 of nitric acid, and 200 of water. The action is still excessively slow, frequently requiring months to be complete.

571. **Chromo-aceto-osmic Acid** (VAN DER STRICHT, *Arch. Biol.*, ix, 1889, p. 29; and SCHAFFER, *Zeit. wiss. Mik.*, x, 1893, p. 179).—Objects to be left in it for months, the liquid being changed at first every two days, afterwards less frequently. Structure well preserved.

572. **Arsenic Acid.**—4 per cent. aqueous solution, used at a temperature of 30° to 40° C. (SQUIRE'S *Methods and Formulæ, etc.*, p. 11).

573. **Phloroglucin with Acids** (ANDEER, *Centralb. med. Wiss.*, xii, xxxiii, pp. 193, 579; *Intern. Monatsschr.*, i, p. 350; HAUG, *Zeit. wiss. Mik.*, viii, 1891, p. 8; FERRERI, *ibid.*, ix, 1892, p. 236; *Bull. R. Accad. Med. di Roma*, 1892, p. 67).—This is said to be the most rapid method of any. Phloroglucin by itself is not a solvent of lime salts; its function in the mixture given below is so to protect the organic elements of tissues against the action of the mineral acid that this can be used in a much more concentrated form than would be otherwise advisable.

ANDEER takes a saturated solution in warm water, and adds to it 5 to 50 per cent. of hydrochloric acid. Wash out in running water.

Other acids than hydrochloric may, of course, be taken. See HAUG, *Zeit. wiss. Mik.*, viii, 1891, p. 8, and FERRERI, *Bull. Acad. Med. Roma*, 1892, p. 67, or (for both) fifth edition.

Desilicification.

574. **Hydrofluoric Acid** (MAYER, *Zool. Anz.*, 1881, p. 593).—The objects are brought in alcohol into a glass vessel coated

internally with paraffin. Hydrofluoric acid is then added drop by drop (taking great care to avoid the fumes, which attack mucous membranes with great energy). Small pieces of siliceous sponges will be completely desilicified in a few hours, or at most a day. The tissues do not suffer.

For sponges I find that this dangerous method can be avoided. If well imbedded, sections may be made from them without previous removal of the spicula, which appear to break off sharp before the knife.

Rousseau imbeds the objects in celloidin, as described § 554, then brings the block, in a covered caoutchouc dish, for a day or two into a mixture of 50 c.c. alcohol and 20 to 30 drops of hydrofluoric acid, and washes out the acid with alcohol containing carbonate of lithia in powder.

Bleaching.

575. **Mayer's Chlorine Method** (*Mitth. Zool. Stat. Neapel*, ii, 1881, p. 8).—Put into a glass tube a few crystals of chlorate of potash, add two or three drops of hydrochloric acid, and as soon as the green colour of the evolving chlorine has begun to show itself, add a few cubic centimetres of alcohol of 50 to 70 per cent. Now put the objects (which must have previously been soaked in alcohol of 70 to 90 per cent.) into the tube. They float at first, but eventually sink. They will be found bleached in from a quarter of an hour to one or two days, without the tissues having suffered. Only in obstinate cases should the liquid be warmed or more acid taken. *Sections on slides* may be bleached in this way. Instead of hydrochloric acid nitric acid may be taken, in which case the active agent evolved is oxygen instead of chlorine.

This method serves both for removing natural pigments, such as those of the skin or of the eyes of Arthropods, and also for bleaching material that has been blackened by osmic acid, and, according to renewed experiments of Mayer's, is to be preferred to the peroxide of hydrogen method.

For bleaching chitin of insects, not alcohol but water should be added to the chlorate and acid (Mayer), *Arch. Anat. Phys.*, 1874, p. 321).

See also Mayer in *Zeit. wiss. Mik.*, xxiv, 1907, p. 353 (paraffin sections exposed to the vapour of chlorine water).

Grynfeltt and Mestrezat (*C. R. Soc. Biol.*, lxi, 1906, p. 87) add 2 c.c. of 20 per cent. solution of chloric acid ($HClO_3$) to 15 c.c. of alcohol and put sections (of retina) into it for several hours at 42° C.

576. **Eau de Labarraque. Eau de Javelle** (see §§ 552, 553).—These are bleaching agents. For the manner of preparing a similar solution see *early editions*, or *Journ. de Microgr.*, 1887, p. 154, or *Journ. Roy. Mic. Soc.*, 1887, p. 518. Of course, the method cannot be used for bleaching soft parts which it is desired to preserve.

577. **Peroxide of Hydrogen (Oxygenated Water)** (Pouchet's method, M. Duval, *Précis*, etc., p. 234).—Macerate in glycerin, to which has been added a little oxygenated water (§ 35), 5 to 6 drops to a watch-glass of glycerin. Solger (*Centralbl. med. Wiss.*, xxi, 1883, p. 177) takes a 3 per cent. solution of peroxide. Fürst (*Morph. Arb. Schwalbe*, vi, 1896, p. 529) points out that after a time it macerates.

The method serves both for removing pigments and for bleaching osmic and chromic material.

578. **Peroxide of Sodium** (Carazzi, *Zool. Anz.*, 444, 1894, p. 135).—See *previous editions*.

579. **Peroxide of Magnesium** (Mayer, *Grundzüge*, p. 290).—Use as chlorine, § 575. A slow but delicate method.

580. **Sulphurous Acid.**—Prof. Gilson writes me that he finds alcoholic solution of sulphurous anhydride (SO_2) very convenient for the rapid decoloration of *bichromate objects*. A few drops suffice. Mönckeberg and Bethe (*Arch. mik. Anat.*, liv, 1899, p. 135) obtain the acid by adding to 10 c.c. of a 2 per cent. solution of bisulphate of sodium 2 to 4 drops of concentrated hydrochloric acid. Objects are put into the freshly prepared solution for six to twelve hours.

581. **Permanganate of Potash.**—Alfieri (*Monitore Zool. Ital.*, viii, 1897, p. 57) bleaches celloidin sections of the choroid, etc., for eight to twenty-four hours in a 1: 2000 solution of permanganate of potash, then washes them out for a few hours in a solution of oxalic acid of 1 : 300 strength, or weaker.

582. **Grenacher's Mixture for Eyes of Arthropods and other Animals** (*Abh. nat. Ges. Halle-a.-S.*, xvi; *Zeit. wiss. Mik.*, 1885, p. 244).

Glycerin	1 part.
80 per cent. alcohol	2 parts.

Mix and add 2 to 3 per cent. of hydrochloric acid.

Pigments (*i. e.* those in question) dissolve in this fluid, and so doing form a stain which suffices in twelve to twenty-four hours for staining the nuclei of the preparation.

583. Nitric Acid.—PARKER (*Bull. Mus. Comp. Zool.*, Cambridge, U.S.A., 1889, p. 173) treats sections (of eyes of scorpions) fixed to the slide with SCHÄLLIBAUM's medium, for about a minute with a solution of up to 50 per cent. of nitric acid in alcohol, or, still better, with a 35 per cent. solution of a mixture of equal parts of nitric and hydrochloric acid in alcohol. To make the solution, the acid should be poured slowly into the alcohol (not *vice versâ*), and the mixture kept cool.

JANDER (*Zeit. wiss. Mik.*, xv, 1898, p. 163) takes for removal of pigments SEILER's chromo-nitric acid (§ 570); twelve to forty-eight hours is enough for small objects.

See also under "Arthropoda."

584. Caustic Soda.—RAWITZ (*Leitfaden*, p. 29) dissolves the pigment of the mantle of Lamellibranchia by means of 3 to 9 drops of officinal caustic soda solution added to 15 to 20 c.c. of 96 per cent. alcohol.

CHAPTER XXV.

EMBRYOLOGICAL METHODS.*

585. Artificial Fecundation.—This practice, which affords the readiest means of obtaining the early stages of development of many animals, may be very easily carried out in the case of the Amphibia anura, Teleostea, Cyclostomata, Echinodermata, and many Vermes and Cœlenterata.

In the case of the Amphibia, both the female and the male should be laid open, and the ova should be extracted from the uterus and placed in a watch-glass or dissecting dish, and treated with water in which the testes, or, better, the vasa differentia, of the male have been teased.

Females of Teleostea are easily spawned by manipulating the belly with a gentle pressure; and the milt may be obtained from the males in the same way. (It may occasionally be necessary, as in the case of the Stickleback, to kill the male, and dissect out the testes and tease them.) The spermatozoa of fish, especially those of the Salmonidæ, lose their vitality very rapidly in water; it is, therefore, advisable to add the milt immediately to the spawned ova, then add a little water, and after a few minutes put the whole into a suitable hatching apparatus with running water.

Artificial fecundation of Invertebrates is easily performed in a similar way. For methods of artificial *Parthenogenesis* see Harvey, *Biol. Bull. Wood's Hole,* 1910, p. 269.

---

* The sections in this chapter treating of *Mammalia*, *Aves*, and *Pisces*, closely follow the *Traité des Méthodes Techniques*, Lee et Henneguy, and are due almost entirely to Henneguy. The corresponding parts of the *Grundzüge*, Lee and Mayer, are taken from this work, and therefore also due to Henneguy, which I regret to observe has not always been understood, though duly pointed out in the Preface to the first edition of the *Grundzüge*.

586. Superficial Examination.—The development of some animals, particularly some invertebrates, may be to a certain extent followed by observations of the living ova under the microscope. This may usefully be done in the case of various Teleosteans, such as the Stickleback, the Perch, *Macropodus*, and several pelagic forms, and with *Chironomus*, *Asellus aquaticus*, Ascidians, *Planorbis*, many Cœlenterata, etc.

Some ova of Insecta and Arachnida which are completely opaque under normal conditions become transparent if they are placed in a drop of oil; if care be taken to let their surface be simply impregnated with the oil, the normal course of development is not interfered with (Balbiani).

587. Fixation.—Osmic acid, employed either alone or in combination with other reagents, is an excellent fixing agent for small embryos, but not at all a good one for large ones. It causes cellular elements to shrink somewhat, and therefore brings out very clearly the slits that separate germinal layers, and any channels or other cavities that may be in course of formation.

In virtue of its property of blackening fatty matters, myelin amongst them, it is of service in the study of the development of the nervous system.

Chromic acid is indispensable for the study of the external forms of embryos; it brings out elevations and depressions clearly, and preserves admirably the mutual relations of the parts; but it does not always preserve the forms of cells faithfully, and is a hindrance to staining in bulk.

Picric liquids have an action which is the opposite of that of osmic acid; they cause cellular elements to swell somewhat, and thus have a tendency to obliterate spaces that may exist in the tissues. But notwithstanding this defect, the picric compounds, and especially Kleinenberg's picro-sulphuric acid, are amongst the best of embryological fixing agents.

Schridde (*Zeit. wiss. Mik.*, xxvii, 1910, p. 362) finds Orth's "Formol-Müller" in general the best fixative. Fix for not more than 24 hours, and pass through graded alcohols (20 minutes in each) into absolute (1 to 2 hours), cedar oil, xylol, and paraffin.

Rabl (*Zeit. wiss. Mik.*, xi, 1894, p. 165) recommends for embryos of Vertebrates, and also for other objects, his platinic sublimate, § 76. This serves for a large number of blastoderms and *young* embryos (Pisces, Amphibia, Aves, Mammalia). *Advanced* embryos of Teleostea ought to be fixed in the *warmed* mixture, in order to avoid rupture of the muscles and shrinkage of the chorda.

Some of his best results were obtained by a *not too prolonged* fixation in a mixture of

Platinic chloride, 1 per cent. solution .	1 vol.
Picric acid, saturated aqueous . .	2 vols
Distilled water	7 ,,

Rabl's picro-sublimate mixture has been given § 70. It is recommended especially for somewhat advanced embryos, such as embryo chicks from the third or fourth day, and other embryos of a similar size.

Boveri (*Verh. Phys. Med. Ges. Würzburg*, xxxix, 1895, p. 4), in order to imbed and cut together numbers of ova of Echinoderms, wraps them in pieces of sloughed epidermis of *Cryptobranchus* (of course, other Urodela will do). Sobotta (*Arch. mik. Anat.*, l, 1897, p. 31) takes pieces of amnios of Mammalia.

Sanzo (*Zeit. wiss. Mik.*, xxi, 1904, p. 449) describes an automatic apparatus for fixing material at definite stages.

588. Peter's Double-stain for Yolk and Tissue, see § 224.

589. Removal of Albumen.—The thick layers of albumen that surround many ova are a serious obstacle to the penetration of reagents. Child (*Arch. Entwickelungsmech.*, ix, 1900, p. 587) gives the following as of very general applicability. After fixation (in any way except with chromic acid) the ova are brought through graduated alcohols up to that of 80 per cent., in which they are hardened. They are then brought down again through successive alcohols into water acidified lightly with any acid (except chromic acid), and the albumen is found to become transparent and dissolve.

590. Reconstruction of Embryos from Sections.—To facilitate the study of series of sections, recourse may be had to graphic or plastic reconstruction of the objects.

In simple cases it may be sufficient to adopt the plan described by SCHAFFER (*Zeit. wiss. Mik.*, vii, 1890, p. 342). Careful outlines of the sections to be reconstructed are drawn on tracing paper with the aid of the camera lucida, superposed, and held up against the light for examination by transparence. VOSMAER (*Anat. Anz.*, xvi, 1899, p. 269) draws on plates of celluloid, and sets them up in a rack for examination. KERR (*Quart. Journ. Mic. Sci.*, xlv, 1902, p. 1) draws on plates of ground glass which he afterwards superposes and makes transparent by oil of cloves run in between them. PENSA (*Zeit. wiss. Mikr.*, xxvii, 1910, p. 48) takes sheets of lithographic gelatin. WOODWORTH (*Zeit. wiss. Mik.*, xiv, 1897, p. 15) proceeds as follows: (1) Draw an axial line of the length of the object multiplied by the magnification employed. (2) Measure with a micrometer the greatest diameter of each section. (3) Plot these diameters down transversely on the axial line at distances corresponding to the thickness of the sections multiplied by the magnification. (4) Join the extremities of these diameters; this will give you an outline of the object. (5) Measure off on each section the nearest and farthest limits (from the margin) of the organs to be filled in, and plot them down on the transverse lines (3), and join the points as before, *i. e.* from section to section; this will give you the outlines of the organs.

This process is best applicable to reconstruction from transverse sections, but it can be applied to reconstruction from sections in any plane if the object can be provided with a plane of definition at right angles to the plane of section. This may be established by cutting off one end of the object, or the like (see also *Orientation*, §§ 142, 161).

To make a simple *plastic* reconstruction, camera drawings (or photographs) of the sections (all made at the same magnification) are pasted on pieces of cardboard of a thickness equal to that of the sections multiplied by the magnification employed. Then the parts of the drawings representing the cavities of the objects are cut out with a knife or fret-saw, cutting through the cardboard; and the pieces of fret-work thus obtained are pasted together.

For more elaborate processes of plastic reconstruction (very complicated and seldom necessary) see BORN, "Die Plattenmodellirmethode," in *Arch. mik. Anat.*, 1883, p. 591, and *Zeit. wiss. Mik.*, v, 1888, p. 433;

STRASSER, *ibid.*, iii, 1886, p. 179, and iv, pp. 168 and 330; KASTSCHENKO, *ibid.*, iv, 1887, pp. 235-6 and 353, and v, 1888, p. 173; SCHAPER, *ibid.*, xiii, 1897, p. 446; ALEXANDER, *ibid.*, p. 334, and xv, 1899, p. 446; PETER, *ibid.*, xxii, 1906, p. 530; BORN and PETER, *ibid.*, xv, 1, p. 31; and *Verh. Anat. Ges.*, xiii, 1899, p. 134; JOHNSTON, *Anat. Anz.*, xvi, 1899, p. 261; FOL, *Lehrb.*, p. 35 or *previous editions*; BROMAN, *Anat. Hefte*, xi, 1899, p. 557; PETER, "Die Methoden d. Rekonstruction" (Fischer, Jena, 1906); SCHÖNEMANN, *Anat. Hefte*, xviii, 1901, p. 117; GAGE, *Anat. Record*, i, 1907, p. 167; NEUMAYER, *Festschr. f. Kupffer*, 1899, p. 459; MARK, *Proc. Amer. Acad. Sci.*, xlii, 1907, p. 629 (electric wax-cutter for cutting out plates).

HILL (*Bull. Johns Hopkins Hosp.*, xvii, 1906, p. 114) finds that embryos of mammalia taken from 95 per cent. alcohol and put into caustic potash of 1 per cent. become so transparent that they can be studied without cutting and reconstructing.

Mammalia.

591. Rabbit—Dissection.—For the study of the early stages the ova must be sought for in the *tubæ* a certain number of hours after copulation. The dehiscence of the follicles takes place about ten hours after the first coitus. The *tubæ* and *cornua* having been dissected out should be allowed to cool, and remain until the muscular contractions have ceased. Then, with the aid of fine scissors or a good scalpel, all the folds of the genital duct are carefully freed from their peritoneal investment.

The tubæ are then (if the ova are still within them, which is the case up to the end of the third day after coition) laid out on a long slip of glass, and slit up longitudinally by means of a pair of fine, sharp scissors. By means of needles and forceps the tubal mucosa is spread out so as to smooth out its folds as much as possible, and is carefully looked over with a strong lens or with a lower power of the microscope. When the ova are found, a drop of some "indifferent" liquid is dropped on each, and it is carefully taken up with the point of a scalpel, a cataract needle, or a small pipette. They may be examined in the peritoneal fluid of the mother if the animal has been killed, or in its aqueous humour, or in amniotic liquid, or in blood-serum, or in KRONECKER's or other artificial serum.

If you have not been able to find the ova with the lens or the microscope, scrape off the epithelium of the tubal mucosa with a small scalpel, mix it with a little indifferent liquid, and look for the ova under the microscope by transmitted light.

Another method, employed by Kölliker, consists in injecting solution of Müller or weak osmic acid into the oviduct by means of a small syringe, and collecting the liquid that runs out in a series of watch-glasses in which the ova can very easily be found by the microscope.

The same doe may be made to serve for two observations, at some hours' or days' interval. A longitudinal incision of 8 to 10 centimetres' length is made on the median or a lateral line of the abdomen; an assistant keeps the intestines in their place; a ligature is placed at the base of one of the uterine cornua, beneath the neck, and a second ligature around the mesometrium and mesovarium. The ovary, the tuba, and the cornu of that side are then detached with scissors. The abdomen is then closed by means of a few sutures passing through the muscle-layers and the skin. The animals support the operation perfectly well, and the development of the ova of the opposite side is not in the least interfered with. When it is desired to study these the animal may be killed, or may be subjected to a secondary laparotomy if it be desired to preserve it for ulterior observations.

During the *fourth*, *fifth*, and *sixth* days after copulation the ova of the rabbit are free in the uterine cornua; they are easily visible to the naked eye, and may be extracted by the same manipulations as those of the tubes. After the sixth day they are at rest in the uterus, but have not yet contracted adhesions with the mucosa, so that they can still be extracted whole. At this stage the parts of the cornua where the ova are lodged are easily distinguishable by their peculiar aspect, the ova forming eminences of the size of a pea. The cornua should be cut up transversely into as many segments as there are eminences, care being taken to have the ova in the centre of the segments. You then fix each segment by means of two pins on the bottom of a dissecting dish, with the mesometrial surface downwards and the ovular eminence upwards. The dissecting-dish is then filled up with serum or liquid of Müller, or 0·1 per cent. solution of osmic acid, or Kleinenberg's picro-sulphuric acid, or nitric acid, or acetate of uranium solution. With a small scalpel a longitudinal incision is made on the surface of the ovular eminence, not passing deeper than the muscular layer; the underlying uterine mucosa is then gently dilacerated with two pairs of small forceps, and the ovum set free in the liquid.

From the moment the ova have become adherent to the uterine mucosa they can no longer be extracted whole. The embryo being always situated on the mesomotrial surface, the ovular eminence is opened by a *crucial* incision, and the strip of mucosa to which the embryo remains adherent is fixed with pins on the bottom of the dish. Ed. v. Beneden (see *Arch. de Biol.*, v, fasc. iii, 1885, p. 378) has been able by operating in this way in serum of Kronecker, and keeping the whole at blood temperature, to observe the circulation of the embryo for hours together. (If this be desired to be done, the crucial incision should not be too extended, so as to leave the terminal sinus intact.)

Retterer (*C. R. Soc. de Biol.*, 1887, p. 99) advises that for ova of the seventh day the segment of uterus containing them be opened *on the mesometrial surface*, for at that date no adhesion has yet been contracted with that side. By running in liquid of Kleinenberg by means of a pipette between the ovum and the free surface of the uterus, the ovum may be got away in the shape of a closed vesicle.

For the study of *living eggs* (of Rats and Mice) see Mark and Long, *Cont. Zool. Lab. Mus. Comp. Zool. Harvard Coll.*, 1912, No. 225 (description of constant temperature chamber and circulation slide).

592. Rabbit; **Microscopic Preparations.**—In order to make permanent preparations of the different stages of fecundation and segmentation, v. Beneden (*Arch. de Biol.*, i, 1, 1880, p. 149) brings the living ovum into a drop of 1 per cent. osmic acid on a slide, and thence into solution of Müller (or bichromate of ammonia or solution of Kleinenberg). After an hour the liquid is changed, and the whole is put into a moist chamber, where it remains for two or three days. It is then treated with glycerin of gradually increasing strength, and at last mounted in pure glycerin acidified with formic acid. Ova may be stained after careful washing.

In order to bring out the outlines of blastoderm cells the living ovum may be brought into one third per cent. solution of nitrate of silver. After remaining there for half a minute to two minutes, according to the age of the vesicle, it is brought into pure water and exposed to the light.

After the end of the third day the blastodermic vesicle can be opened with fine needles, and the blastoderm washed, stained, or impregnated with gold, and mounted in glycerin or balsam.

For embryonic areas and more advanced embryos KÖLLIKER recommends putting the ovum into 0·5 per cent. solution of osmic acid until it has taken on a somewhat dark tint, which happens in about an hour, and then treating it with successive alcohols for several hours. If the ovum be adherent to the uterine mucosa the portion of the membrane to which it is fixed should be left, stretched out with pins, in 0·1 per cent. solution of osmic acid for from four to six hours. The blastodermic vesicle can then easily be removed, and further treated as before. For sections KÖLLIKER fixes with osmic acid. v. BENEDEN treats the ova for twenty-four hours with 1 per cent. solution of chromic acid, then washes well, and brings them through successive alcohols. Chromic acid has the advantage of hardening thoroughly the vesicle, and maintaining at the same time the epiblast cells perfectly adherent to the zona pellucida. v. BENEDEN also recommends the liquid of Kleinenberg. HENNEGUY writes that he frequently employs it for embryonic areas and embryos of various ages, always with excellent results. Fol's modification of the liquid of Flemming, and Ranvier and Vignal's osmic acid and alcohol mixture (§ 36) also give excellent results. For staining, HENNEGUY recommends borax-carmine, or Delafield's hæmatoxylin for small embryos; for large ones he found that his acetic acid alum-carmine was the only reagent that would give a good stain in the mass.

For sections imbed in paraffin.

See also WEYSSE, *Proc. Amer. Acad. Arts. and Sci.*, 1894, p. 285 (blastodermic vesicle of *Sus scrofa*); SOBOTTA, *Arch. mik. Anat.*, xlv, 1895, p. 15 (ovum of the Mouse; fixation in FLEMMING'S weak mixture, sections stained with BENDA'S iron hæmatoxylin), and *Anat. Hefte*, 1 Abth., viii, 1897, p. 476 (Rabbit; fixation with liquid of Flemming or picro-sublimate with 2 per cent. acetic acid); BONNET, *ibid.*, ix, 1897, p. 426 (Dog; fixation in sublimate); SELENKA, *Stud. Entw. d. Thiere*, Wiesbaden, 1883, p. 5, and 1887, p. 107 (picrosulphuric acid for the mouse, and picric acid with $\frac{1}{10}$ per cent. of chromic acid for *Didelphys*); KEIBEL, *Morph. Arb.*, ii, 1893, p. 11 (*Sus scrofa*); NEUMAYER, *Festschr. f. Kupffer*, 1899, p. 458

(embryos of the sheep best fixed in Carnoy's acetic acid, alcohol, and chloroform, § 85); Winiwarter, *Arch. Biol.*, xvii, 1900, p. 39 (mixture of 50 parts saturated sublimate in salt solution, 50 parts alcohol, 20 of 1 per cent. platinum chloride, and 5 of acetic acid); Spee, *Encycl. Mik. Techn.*, 1910, p. 353 (cornua of *Cavia* fixed for 12 to 24 hours in sublimate, and put into 0·5 per cent. osmic acid till light brown, then into iodine alcohol, in which the osmium is reduced); Widakowich, *Zeit. wiss. Zool.*, xciv, 1909, p. 243 (*Mus rattus*, fixation in Zenker's mixture, or 2 parts of alcohol of 80 per cent. with 1 of formol; also instructions for dissection).

Aves.

593. Superficial Examination.—Instructions on this head are given in Foster and Balfour's *Elements of Embryology*. The following is of more recent publication.

If it be desired to observe a living embryo by transmitted light, the egg should be opened under salt solution, as described below. A little of the white is then removed through the window, the egg is lifted out of the liquid, and a ring of gummed paper is placed on the yolk so as to surround the embryonic area. As soon as the paper adheres to the vitelline membrane, which will be in a few minutes, a circular incision is made in the blastoderm outside the paper ring. The egg is put back into the salt solution, and the paper ring removed, carrying with it the vitelline membrane and the blastoderm, which may then be brought into a watch-glass or on to a slide and examined under the microscope (Duval).

594. Gerlach's Window Method (*Nature*, 1886, p. 497).—Remove with scissors the shell from the small end of the egg; take out a little white by means of a pipette; the blastoderm will become placed underneath the window just made, and the white that has been taken out may be replaced on it. Paint the margins of the window with gum mucilage, and build up on the gum a little circular wall of cotton wool; place on it a small watch-glass (or circular cover glass), and ring it with gum. When the gum is dry the cover is further fixed in its place by means of collodion and amber varnish, and the egg is put back in its normal position in the incubator. The progress of the development may be followed up to the fifth day through the window.

A description of further developments of this method, with figures of special apparatus, will be found in *Anat. Anz.*, ii, 1887, pp. 583, 609.

See also PATON, *Journ. Exper. Zool.*, xi, 1911, p. 469 (cultivation of the embryo *in vitro*).

595. Preparation.—During the first twenty-four hours of incubation it is extremely difficult to separate the blastoderm from the yolk, and they should be fixed and hardened together.* In later stages, when the embryo is conspicuous, the blastoderm can easily be separated from the yolk, which is very advantageous. To open the egg, lay it on its side and break the shell at the broad end by means of a sharp rap; then carefully remove the shell bit by bit by breaking it away with forceps, working away from the broad end until the blastoderm is exposed. The egg should be opened in salt solution, then lifted up a little, so as to have the blastoderm above the surface of the liquid; the blastoderm is then treated with some fixing solution dropped on it from a pipette (1 per cent. solution of osmic acid, or Ranvier and Vignal's osmic acid and alcohol mixture, iodised serum, solution of Kleinenberg, 10 per cent. nitric acid, etc.). By keeping the upper end of the pipette closed, and the lower end in contact with the liquid on the blastoderm, the blastoderm may be kept well immersed for a few minutes, and should then be found to be sufficiently fixed to be excised. (Of course, if you prefer it, you can open the egg in a bath of any fixing liquid [10 per cent. nitric acid being convenient for this purpose] of such a depth as to cover the yolk; and having exposed the blastoderm, leave it till fixed [fifteen to twenty minutes]; but I think the procedure above described will generally be found more convenient.)

The egg is put back into the salt solution, and a circular incision made round the embryonic area. The blastoderm may then be floated out and got into a watch-glass, in which it may be examined, or may be brought into a hardening liquid.

---

* ANDREWS (*Zeit. wiss. Mik.*, xxi, 1904, p. 177) separates the blastoderm at this stage by injecting picro-sulphuric acid (not any rapidly acting fixative) firstly between the blastoderm and the vitelline membrane, so as to separate the two above, and then between the blastoderm and the yolk, so as to free the blastoderm below and float it up. This done, the membrane may be incised and the blastoderm removed. The injection is best done with a pipette having a fine point bent upwards.

Before putting it into the hardening fluid, the portion of vitelline membrane that covers the blastoderm should be removed with forceps and shaking.

Fixation in 10 per cent nitric acid has the advantage of greatly facilitating the separation of the blastoderm. The acid should be allowed to act for ten minutes, after which it is well to bring the preparation into 2 per cent. solution of alum (*cf.* HOFMANN, *Zeit. wiss. mik.*, x, 1893, p. 485). MITROPHANOW (*Anat. Hefte*, xii, 1899, p. 200) fixes with nitric acid of 3 per cent., SUSCHKIN (*Nouv. Mém. Soc. Nat. Moscow*, xvi, 1899, p. 34) with sublimate; FISCHEL (*Morph. Jahrb.*, xxiv, 1896, p. 371) with Rabl's platino-sublimate, § 76 (embryos of the duck); PATTERSON (*Biol. Bull. Wood's Hole*, xiii, 1907, p. 252) with picro-sulphuric acid containing 8 per cent. of acetic acid, for an hour (ova of *Columba*); HOSKINS (*Kansas Univ. Sci. Bull.*, iv, 1907, p. 176), after removing shell, for 5 to 15 minutes in a mixture of 3 parts of 10 per cent. formol with 1 of 10 per cent. nitric acid, and then excises the embryo.

In order to counteract the turning up of the edges of the blastoderm that generally happens during the process of hardening, it is well to get the blastoderm spread out on the *convex* surface of a watch-glass, and leave it so during the hardening.

For hardening HENNEGUY prefers the osmic acid and alcohol mixture of Ranvier and Vignal, or Flemming's mixture followed by successive alcohols.

Stain and imbed by the usual methods.

Up to about the fiftieth hour embryos may be mounted entire in glycerin or balsam.

596. M. DUVAL'S **Orientation Method** (*Ann. Sc. Nat.*, 1884, p. 3).—In the early stages of the development of the ova of Aves, before the appearance of the primitive streak, it is difficult to obtain a correct orientation of the hardened cicatricula, so as to be able to make sections in any desired direction. DUVAL, starting from the fact that during incubation the embryo is almost always found to be lying on the yolk in such a position that the big end of the egg is to the left, and the little end to the right of it, marks the position of the blastoderm in the following way.

With a strip of paper 5 millimetres wide and 50 millimetres long you construct a sort of triangular bottomless box. You lay this on the yolk, enclosing the cicatricula in such a position that the base of the triangle corresponds to what will be the anterior region of the embryo, and its apex to the posterior region; that is to say, if the big end of the egg is to your left, the apex of the triangle will point towards you. You now, by means of a pipette, fill the paper triangle with 0·3 per cent. solution of osmic acid. As soon as the preparation begins to darken you put the whole egg into weak chromic acid, remove the white, and put the rest into clean chromic acid solution for several days. After hardening you will find on the surface of the yolk a black triangular area, which encloses the cicatricula and marks its position; you cut out this area with scissors and a scalpel, and complete the hardening with chromic acid and alcohol.

See also the method of HIROTA, *Journ. Roy. Mic. Soc.*, 1895, p. 118.

597. KIONKA'S **Orientation Method** (*Anat. Hefte*, 1 Abth., iii, 1894, p. 414).—Open the egg under salt solution, free it from the shell and albumen, and mark the poles by sticking into it, at about a centimetre from the blastoderm, two hedgehog spines, the one at the obtuse end being marked with a red thread. Put the whole for ten minutes into water at 90° C., then bring into 70 per cent. alcohol, and after twenty-four hours cut out the blastoderm and a little yolk round it in the shape of an isosceles triangle, whose base marks the anterior end of the blastoderm. Paraffin sections stained with borax-carmine, washed out with acid alcohol containing one drop of concentrated solution of Orange G for each 5 c.c., which stains the yolk.

598. VIALLETON'S **Method** (*Anat. Anz.*, vii, 1892, p. 624).—Egg opened in salt solution, blastoderm excised and removed to a glass plate, then treated with 1 per cent. nitrate of silver solution, washed with water, and put into 70 per cent. alcohol for six to twelve hours in the dark. Borax-carmine, alcohol, damar.

599. BÖHM and OPPEL (*Taschenbuch*, 1896, p. 80) fix ova with fairly large embryos in a mixture of 20 parts 3 to 5 per cent. nitric acid and 1 to 2 parts 1 per cent. silver nitrate.

Reptilia.

600. General Directions.—The methods described above for birds are applicable to reptiles. During the early stages the blastoderm should be hardened *in situ* on the yolk; later the embryo can be isolated, and treated separately.

Böhm and Oppel (*Taschenbuch*, 1900, p. 186) remove the shell under salt solution, fix in sublimate with 20 per cent. acetic acid, or in Lo Bianco's chromo-sublimate (§ 72), then remove the blastoderm and bring it into alcohol.

601. Special Cases.—Mitsukuri (*Journ. Coll. Sc. Japan*, vi, 1894, p. 229) fixes embryos of tortoises chiefly with picro-sulphuric acid. To study the blastoderm he removes the whole of the shell and as much as possible of the albumen, marks the place where the blastoderm lies with a hair, brings the whole, with the blastoderm uppermost, into the fixative, and after a few hours cuts out the blastoderm and further hardens it by itself. Young embryos generally adhere to the shell and can, therefore, be fixed in a piece of it made to serve as a watch-glass, then after half-an-hour can be removed from it and further hardened alone. If the embryonal membranes have been formed, the shell may be scraped away at some spot and there treated with picro-sulphuric acid until a small hole is formed; then by working away from this spot, by means of scraping and dropping acid on to it, the whole of the shell may be removed.

Will (*Zool. Jahrb., Abth. Morph.*, vi, 1892, p. 8) opens ova of *Platydactylus* in the fixative (chiefly chromic acid, or chromo-aceto-osmic acid with very little osmic acid) and hardens the embryos on the yolk; so also for *Cistudo* and *Lacerta* (1893 and 1895). Mehnert (*Anat. Anz.*, xi, 1895, p. 257) does not approve of these methods; for his own see *Morph. Arb. Schwalbe*, i, 1891, p. 370.

Gerhardt (*Anat. Anz.*, xx, 1901, p. 244) fixes ova of *Tropidonotus* for 24 hours in Nowak's mixture, § 112.

Ballowitz (*Entwickl. d. Kreuzotter*, 1903, p. 19) first fixes segments of the uterus, each containing an ovum, for 1 or 2 hours, then tears them open with forceps, isolates the ova, and puts them into fresh fixative, and thence into alcohol of 40 per cent.

Nicolas (*Arch. Anat. Mic.*, 1900, p. 457) finds the best fixative for ova of the slow-worm, as for other large ova, is Bouin's picro-formol (§ 110).

See also Perenyi, § 48, and *Zool. Anz.*, 1888, pp. 139 and 196, and other methods in *early editions*.

Amphibia.

602. Preliminary.—In order to prepare ova for section-cutting, it is essential to begin by removing their thick coats of albumen. This may be done by putting them for two or three days into 1 per cent. solution of chromic acid, and shaking well; but ova thus treated are very brittle, and do not afford good sections. A better method is that described by Whitman (*Amer. Natural.*, xxii, 1888, p. 857), and by Blochmann (*Zool. Anz.*, 1889, p. 269). Whitman puts the fixed eggs into a 10 per cent. solution of sodium hypochlorite diluted with 5 to 6 volumes of water, and leaves them there till they can be shaken free, which happens (for *Necturus*) in a few minutes. Blochmann takes *eau de Javelle* (potassium hypochlorite), and dilutes it with 3 to 4 volumes of water, and agitates the eggs previously fixed with solution of Flemming, for fifteen to thirty minutes in it. See also § 589.

Lebrun (*La Cellule*, xix, 1902, p. 316) advises fixing ova of Anura for not less than 1½ hours in liquid of Gilson, § 69. The outer envelopes are then hard, and may be easily incised and the ovum extracted by pressing on the pole opposite to the incision. The operation should *not* be delayed until after hardening in alcohol. Similarly (*ibid.*, xx, 1902, p. 12), for Urodela.

Guyer (*Amer. Nat.*, xli, 1907, p. 400) finds it suffice to roll the ova (either fresh or fixed, but before bringing into alcohol) on blotting paper.

603. Imbedding.—A great difficulty with the ova of Amphibia lies in their becoming extremely brittle on imbedding in paraffin. Carnoy and Lebrun (*La Cellule*, xii, 1897, p. 212) fix ovaries or ovarian ova for fifteen minutes to three quarters of an hour (but see last §) in Gilson's mercuro-nitric fluid, § 69, and preserve them in 80 per cent. alcohol. To imbed, they are brought for a quarter of an hour into 95 per cent. alcohol, five minutes in absolute alcohol, then into a mixture of alcohol and chloroform in equal parts, and as soon as they sink in that they are put into pure chloroform. Paraffin is added to the chloroform, enough to about double the volume of the whole, and the whole is put for about three hours into a stove at 35° C. Lastly, the ova are put *for not more than five minutes* into a bath of pure paraffin at 52° C.

Later (*ibid.*, xix, 1902, p. 317) LEBRUN explains that it is important not to dehydrate completely with absolute alcohol; the ova should be left in alcohol of 96 per cent. until chloroform can be added without the mixture becoming turbid, and a second bath of clean paraffin should be added.

See also MORGAN, *Devel. of the Frog's Egg*, New York, 1897, p. 171.

604. **Siredon.**—The ova are easier to prepare than those of the Anura, because the yolk is separated from the albuminous layer by a wide space filled with a liquid that is not coagulated by reagents. Put the eggs for a few hours into picro-sulphuric acid, then pierce the inner chorion with fine scissors or needles, and gently press out the ovum. Harden in alcohol.

FICK (*Zeit. wiss. Zool.*, lvi, 1893, p. 529) uses a mixture of 250 parts of 1 per cent. chromic acid, 1 of acetic acid, and 750 of water.

605. **Triton** (SCOTT and OSBORN, *Quart. Journ. Mic. Soc.*, 1879, p. 449).—The albumen is here present in the form of several concentric coats, which are very delicate. Incise each of them separately with fine scissors, turn out the ovum, and fix it in solution of Kleinenberg.

HERTWIG (*Jen. Zeit. Naturw.*, 1881–2, p. 291) puts the eggs into a mixture of equal parts of 2 per cent. acetic acid and 0·5 per cent. chromic acid. After ten hours he incises the membranes, opening one end of the inner chorion, and turns out the embryos and brings them into successive alcohols.

MICHAELIS (*Arch. mik. Anat.*, xlviii, 1896, p. 528) fixes ova, with their envelopes, in a mixture of concentrated sublimate solution and concentrated picric acid, twenty parts each, glacial acetic acid 1, and water 40, but removes the envelopes before bringing into alcohol.

606. Salamandra (RABL, *Morphol. Jahrb.*, xii, 2, 1886, p. 252).—For his more recent methods see § 587.

GRÖNROSS (*Anat. Anz.*, xiv, 1898, p. 461) fixes the ova with a mixture of 50 parts each of saturated sublimate and 0·5 per cent. chromic acid with one part of acetic acid.

607. Rana (O. HERTWIG, *Jen. Zeit. Naturw.*, xvi, 1883, p. 249).—The ova are thrown into nearly boiling water (90° to 96° C.) for five or ten minutes. The albuminous envelope of the ovum is then cut open, and the ovum extracted under water. The ova are then brought into 0·5 per cent. chromic acid for not more than twelve hours, or into alcohol of 70, 80, and 90 per cent. Chromic acid makes ova brittle and attacks the pigment, whilst alcohol preserves it, which is frequently important for the study of the germinal layers.

MORGAN (*Amer. Nat.*, xxv, 1891, p. 759, and *Devel. of the Frog's Egg*, 1897, p. 171) has the following. During the periods in which it is difficult or impossible to remove the inner jelly-membrane the eggs can be freed as follows: Each egg is cut out with scissors from the general jelly-mass, and put for from one to twelve hours into saturated solution of picric acid in 70 per cent. alcohol containing 2 per cent. of sulphuric acid. Wash in several changes of alcohol of 70 per cent. About the second day in this the inner membrane begins to swell, and on the third or fourth day may be pierced by a needle, and the egg removed and placed in 80 per cent. alcohol (see also WHITMAN, *Meth. of Research*, p. 156).

SCHULTZE (*Arch. mik. Anat.*, lv, 1899, p. 174) removes with scissors the outer layers of albumen, and puts the ova for five minutes in 2 per cent. formol warmed to 75° or 80° C. The membrane left on the ova then rises up sufficiently to allow the ova to be got out with needles.

See also BORN (*ibid.*, xliii, 1894, p. 1).

KING (*Journ. Morph.*, xvii, 1901, p. 295, and xix, 1908, p. 370) fixes (for a few minutes) the spawn (of *Bufo*) in sublimate (saturated with 5 per cent. of acetic acid), or in Flemming, Zenker, or Hermann, brings into alcohol, first of 50 and then 80 per cent., and removes the jelly after a few days.

BLES (*Trans. Roy. Soc. Edinburgh*, xli, 1905, p. 792) takes for ova formol of 10 per cent., but for embryos and larvæ the mixture given § 109.

Bouin takes for larvæ of *Rana* the formol-sublimate mixture § 112.

608. Sulphate of Copper Liquid (Fol, *Lehrbuch*, p. 106, after Remak and Goette); for hardening ova of Amphibia:

2 per cent. solution of sulphate of copper .	50 c.c.
Alcohol of 25 per cent.	50 ,,
Rectified wood vinegar	35 drops.

Pisces.

609. Teleostea in General.—The ova of many of the bony fishes can be studied by transmitted light in the living state; but those of the Salmonidæ must be hardened and removed from their envelopes for the study of the external forms of the embryo.

To this end they may be put for a few minutes into water containing 1 to 2 per cent. of acetic acid, and thence into 1 per cent. chromic acid. After three days the capsule of the ovum may be opened at the side opposite to the embryo, and be removed with fine forceps. The ovum is put for twenty-four hours into distilled water, and then into successive alcohols. Embryos thus prepared show no deformation, but the vitellus rapidly becomes excessively hard and brittle, so as greatly to interfere with section-cutting.

The following processes give good results as regards section-cutting.

Put the ova for a few minutes into 1 per cent. osmic acid; as soon as they have taken on a light brown colour bring them into Müller's solution. Open them therein with fine scissors—the vitellus, which immediately coagulates on contact with air, dissolves, on the contrary, in Müller's solution—and the germ and cortical layer can be extracted from the capsule of the ovum. They should be left in clean Müller's solution for a few days, then washed with water for twenty-four hours, and brought through successive alcohols.

Another method (Henneguy) is as follows: The ova are fixed in solution of Kleinenberg containing 10 per cent. of acetic acid. After ten minutes they are opened in water containing 10 per cent. of acetic acid, which dissolves the vitellus. The embryos are put for a few hours into pure solution of Kleinenberg, and are then brought through alcohol of gradually increasing strength.

Child (quoted from Sumner, *Mem. New York Acad. Sci.*, ii, 1900, p. 78) fixes for about a minute in sublimate with 10 per cent. of acetic acid, and brings into formalin of 10 per cent., which is said to give a good fixation of the embryo without the yolk becoming hard.

610. Kollmann's Fixative (Kollmann, *Arch. Anat. Phys.*, 1885, p. 296).

Bichromic of potash	5 per 100.
Chromic acid	2 ,,
Concentrated nitric acid	2 ,,

For ova of Teleostea. Fix for twelve hours, wash with water for twelve hours, then remove the chorion, and put the ova into 70 per cent. alcohol.

611. Rabl's Method see § 587; for Kowalewsky's see *Zeit. wiss. Zool.*, xliii, 1886, p. 434, or *Third Edition.*

612. Salmonidæ.—Henneguy's methods have been given, § 609.

Kopsch (*Arch. mik. Anat.*, li, 1897, p. 184), on the suggestion of Virchow, fixes embryos for five or ten minutes in a mixture of 1 part of chromic acid to 50 of glacial acetic acid and 450 of water, then removes into chromic acid of 1 : 500, and as soon as may be removes the capsule and yolk *under salt solution*, and completes the hardening in the chromic acid or the saturated sublimate solution.

Similarly, Behrens (*Anat. Hefte*, x, 1898, p. 233).—He opens the ova in the salt solution from the *antipolar* side, and frees the embryo from the yolk that remains by blowing the latter away with a fine-pointed glass tube.

Similarly also Sobotta (*ibid.*, 1902, p. 579).

Gudger (*Proc. U.S. Nation. Mus.*, xxix, 1906, p. 448) fixes blastoderms in fresh liquid of Perényi, which does not make the yolk too hard; later stages in Worcester's liquid (9 parts of saturated solution of sublimate in formol of 10 per cent. and 1 part of acetic acid), for half an hour to an hour, and brings gradually into alcohol of 70 per cent.

Bouin (*C. R. Soc. Biol.*, lv, 1903, p. 1691) fixes for 36 to 48 hours in picro-formol.

Rabl-Rückhard's Method (*Arch. Anat. Entw.*, 1882, p. 118).—Fix in 10 per cent. nitric acid for fifteen minutes. Remove the membranes to avoid deformation of the embryos, and put the ova back into the acid for an hour. Wash out in 1 to 2 per cent. solution of alum for an hour and harden in alcohol.

Modification of this method by GORONOWITSCH (see *Morph. Jahrb.*, x, 1884, p. 381).

613. Selachia.—BEARD (*Anat. Anz.*, xviii, 1900, p. 556) has found that the best fixatives for embryos of *Raja* are Rabl's picro-platinic mixture, § 587 and sublimate.

Living embryos can be observed by scraping the shell thin with a knife (KASTSCHENKO, *Anat. Anz.*, iii, 1888, p. 445, and HIS, *Arch. Anat. Phys. Anat. Abth.*, 1897, p. 3). See also BRAUS, *Morph. Jahrb.*, xxxv, 1906, p. 250.

614. Amphioxus.—SOBOTTA (*Arch. mik. Anat.*, 1, 1897, p. 20) fixes for twenty-four hours in liquid of Flemming; HATSCHEK (*Arb. Zool. Inst. Wien.*, iv, 1881) in picro-sulphuric acid. Impregnation takes place in the evening, and segmentation is completed during the night.

LEGROS (*Grundzüge*, LEE and MAYER, 1910, p. 288) fixes ova and embryos in equal parts of formol and Flemming. Sublimate is not good; Rabl's mixtures are better. Larvæ and young animals ought first to be anæsthetised with cocain in sea-water. After fixation they should remain only for as short a time as possible in alcohol.

CERFONTAINE (*Arch. Biol.*, xxii, 1906, p. 287) fixes with Flemming or Hermann. For study of ova *in toto* he orients them on a slide in clove-oil-collodion which he sets with chloroform, and adds balsam. For sectioning, he orients in the same way on a layer of paraffin spread on a cover glass, and imbeds the whole in paraffin.

615. Pelagic Fish Ova.—WHITMAN (*Amer. Natural*, xvii, 1883, pp. 1204–5; and *Methods of Research*, etc., p. 152).—Fix by treatment first for five to ten minutes with a mixture of equal parts of sea-water and $\frac{1}{2}$ per cent. osmic acid solution, and then for one or two days with a solution (due to Eisig) of equal parts of 0·25 per cent. platinum chloride and 1 per cent. chromic acid. Prick the membrane before transferring to alcohol. See also AGASSIZ and WHITMAN, in *Proc. Amer. Acad. Arts and Sciences*, xx, 1884; and COLLINGE, *Ann. and Mag. Nat. Hist.*, x, 1892, p. 228.

RAFFAELE (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 169) fixes chiefly with liquid of Hermann (1 to 2 days), or with a mixture of Mingazzini (absolute alcohol 1, acetic acid 1, saturated sublimate solution in water 2).

HEINKE and EHRENBAUM (*Wiss. Meeresunt. Komm. Wiss. Unt. D.*

Meere, iii, Heligoland, 1900, pp. 205 and 213) prefer formol with 39 vols. of sea-water.

Tunicata.

616. **Ova.**—Davidoff (*Mitth. Zool. Stat. Neapel*, ix, 1, 1889, p. 118) fixes the ova of *Distaplia* with a mixture of 3 parts of saturated solution of corrosive sublimate and 1 of glacial acetic acid for from half an hour to an hour; or with a mixture of 3 parts of saturated solution of picric acid and 1 of glacial acetic acid for three to four hours; then 70 per cent. alcohol.

Castle (*Bull. Mus. Harvard Coll.*, xxvii, 1896, p. 213) advises for ova of *Ciona* liquid of Perényi for twenty minutes, followed by 70 per cent. alcohol for twenty-four hours, and for the larvæ picro-nitric acid.

617. **Test-Cells of Ascidians** (Morgan, *Journ. of Morphol.*, iv, 1890, p. 195).—Tease fresh ovaries in very weak osmic acid, wash in distilled water, treat for half an hour with 1 per cent. silver nitrate, wash for half an hour in 2 per cent. acetic acid and reduce in sunlight. Imbed in paraffin. By this process the *limits* of the follicle cells are demonstrated.

618. **Buds.**—Pizon (*Ann. Sc. Nat.*, xix, 1893, p. 5) studies the gemmation of the composite Ascidians either on entire corms, which he first bleaches with peroxide of hydrogen and then stains; or by making sections, after anæsthetising the colonies with cocain of 1 : 1000, fixing in glacial acetic acid or picro-sulphuric or liquid of Flemming, and staining *in toto* with borax carmine or alum carmine, or with a strong solution of methylen blue in alcohol of 90 or 100 per cent. (after Bernard, *ibid.*, ix, 1890, p. 97).

Ritter (*Journ. of Morph.*, xii, 1896, p. 150) recommends for fixing *Perophora* and *Goodsiria* picro-sulphuric acid.

Bryozoa.

619. **Statoblasts.**—Braem (*Bibl. Zool., Chun and Leuckart*, 6 Heft, 1890, p. 95) fixest statoblasts of *Cristatella* with hot concentrated solution of sublimate for ten minutes, brings them into water and there incises them with a razor, and after half an hour passes them gradually into alcohol. He stains with picro-carmine.

Mollusca.

620. Cephalopoda (Ussow, *Arch. de Biol.*, ii, 1881, p. 582).—Segmenting ova are placed in 2 per cent. solution of chromic acid for two minutes, and then in distilled water, to which a little acetic acid (one drop to a watch-glassful) has been added, for two minutes. If an incision be now made into the egg-membrane the yolk flows away and the blastoderm remains; if any yolk still cling to it, it may be removed by pouring away the water and adding more.

Watasé (*Journ. of Morphol.*, iv, 1891, p. 249) kills the ova in the macerating mixture of the Hertwigs (§ 534), and as soon as the blastoderm turns white and opaque removes it under dilute glycerin. Treatment with liquid of Perényi is recommended for surface views.

Vialleton (*Ann. Sc. Nat.*, vi, 1887, p. 168) brings ovarian ova of Sepia into a freshly prepared mixture of picro-sulphuric acid and 2 per cent. solution of bichromate of potash in equal parts, and after one or two minutes incises them in the equator, fixes for an hour and a half in picro-sulphuric acid the halves that contain the formative vitellus, separates this from the nutritive vitellus with a spatula, spreads it out, and hardens it in alcohol of 70 to 90 per cent. He fixes entire ova in liquid of Flemming or osmic acid.

Korschelt (*Festschrift Leuckart*, Leipzig, 1892, p. 348) fixes advanced embryos of Loligo in liquid of Flemming, sublimate, picro-sulphuric acid, or 0·2 per cent. chromic acid. This last is specially good for young embryos if it is washed out with many changes of picric acid.

Faussek (*Mitth. Zool. Stat. Neapel*, xiv, 1900, p. 83) recommends picro-nitric acid. Fix in this, harden in alcohol, bring the ova, still in their albumen, into hæmalum, stain for 24 hours, wash in 1 per cent. alum solution for 24 hours, when the albumen will be found softened so that the ova can easily be extracted.

621. Gastropoda (Henneguy).—Ova of *Helix* may be fixed for from four to six hours in Mayer's picro-nitric acid. The carbonate of lime that encrusts the external membrane is thus dissolved, and the albuminous coat of the egg is coagulated. The egg is opened with needles, the albumen comes away in bits, and the embryo can be removed.

Henchman (*Bull. Mus. Comp. Zool., Harvard*, xx, 1890, p. 171) fixes ova of *Limax* with 0·33 per cent. chromic acid, or with liquid of Perényi. It is best to remove only the outer envelope before putting into the chromic acid, the inner membrane being removed after two or three minutes therein. Where Perényi is used the membranes must be removed first, as the albumen will else coagulate in such a way as to prevent the removal of the embryos.

Meisenheimer (*Zeit. wiss. Zool.*, lxii, 1896, p. 417) dissects out the embryos of *Limax* and fixes them with picro-sulphuric acid or concentrated sublimate. Advanced embryos are first got into extension by means of 2 per cent. cocaine, or are rapidly killed with hot sublimate.

Schmidt (*Entw. Pulmonaten*, Dorpat, 1891, p. 4) fixes the ova *in toto* with concentrated sublimate, and dissects them out afterwards.

Similarly Kofoid (*Bull. Mus. Harvard Coll.*, xxvii, 1895, p. 35). Or, preferably, the ova are put into salt solution, the shell removed, the albumen removed with a pipette full of salt solution, which dissolves it; the ova are then fixed for one minute in Fol's modification of liquid of Flemming, and brought direct into Orth's picro-lithum-carmine. See also Linville, *ibid.*, 1900, p. 215, who adopts this method of shelling, but prefers fixing in acetic-acid-sublimate, or liquid of Perényi.

Heyder (*Zeit. wiss. Zool.*, xciii, 1909, p. 92), before imbedding embryos of *Arion* that have been fixed with sublimate, treats them for an hour or two with carbonate of soda of one tenth to one fifteenth per cent., which makes the stomach and intestine less brittle.

Holmes (*Journ. of Morph.*, 1900, p. 371) teases the egg-capsules of *Planorbis* in nitrate of silver of $\frac{3}{4}$ per cent., exposes to sunlight until the cell-limits come out, rinses with 0·2 per cent. hyposulphite of soda, puts for a few minutes into picric acid, and then through alcohol into balsam.

See also Washburn, *Amer. Anat.*, xxviii, 1894, p. 528 (liquid of Flemming or 0·3 per cent. chromic acid, or 1 per cent. osmic acid, followed by liquid of Merkel).

Conklin (*Journ. of Morph.*, xiii, 1897, p. 7) fixs ova of *Crepidula* for fifteen to thirty minutes in picro-sulphuric acid, and stains with dilute acidified hæmatoxylin of Delafield.

KOSTANECKI and WIERZEJSKI (*Arch. mik. Anat.*, xlvii, 1896, p. 313) fix the spawn of *Physa fontinalis* either in 1½ to 2 per cent. nitric acid, or in "sublimate and 3 per cent. nitric acid in the proportion of 2 : 1," and bring through successive alcohols. They imbed entire ova in paraffin, but isolated embryos in celloidin.

622. CHITON, see METCALF, *Stud. Biol. Lab. Johns Hopkins Univ.*, v, 1893, p. 251. (Ova with young embryos put for 20 to 45 seconds into *eau de Labarraque*, then into water, in which the chorion swells and can easily be removed.)

623. **Lamellibranchiata.** - STAUFFACHER (*Jena Zeit.*, xxviii, 1893, p. 196) fixes embryos of *Cyclas* in sublimate, stains with hæmalum, and cuts in paraffin.

LILLIE (*Journ. of Morph.*, x, 1895, p. 7) fixes ova of *Unio* for ten to twenty minutes in liquid of Perényi, and preserves them in 70 per cent. alcohol, or advanced embryos with liquid of Merkel or sublimate, larvæ with 0·05 to 0·1 per cent. osmic acid, preserving them in glycerin. Glochidia may be cut with the shell in paraffin of 58° melting-point; they may be anæsthetised with chloral hydrate before fixing.

Arthropoda.

624. **Fixation of Ova.**—In many cases the ova of Arthropods are best fixed by heat (§ 13). This may be followed either by alcohol or some watery hardening agent. If it be desired to avoid heating, picro-nitric acid may be tried.

625. **Removal of Membranes.**—It may often be advisable not to attempt to remove them, but to soften them with *eau de Javelle* or *eau de Labarraque* (see § 553).

MORGAN (*Amer. Natural.*, xxii, 1888, p. 357) recommends (for the ova of *Periplaneta*) *eau de Labarraque* diluted with five to eight volumes of water, and slightly warmed. This will soften the chitin membranes sufficiently in thirty to sixty minutes, if employed before fixing. Fixed ova take longer. The fluid must, of course, not be allowed to penetrate into the interior of the ovum.

626. HENKING'S **Methods** (*Zeit. wiss. Mik.*, viii, 1891, p. 156).—HENKING generally kills ova by plunging them into hot water, or by pouring hot water on to them in a watch-glass, and then removing into 70 per cent. alcohol.

He thinks that *eau de Javelle* for softening membranes is best avoided. They should either be dissected away or left *in situ*, and cut with the rest of the egg, according to the nature of the case. To avoid brittleness of the yolk proceed as follows: After fixing and treating with alcohol, prick the chorion and stain with borax-carmine. Put the stained ova for twelve hours into a mixture containing 20 c.c. of 70 per cent. alcohol, one drop of concentrated hydrochloric acid, and a knife pointful of pepsin (it is not necessary that all the pepsin should be dissolved). The ova may then be treated with alcohol, oil of bergamot, and paraffin, and (with some exceptions, amongst which is *Bombyx mori*) will be found to cut without crumbling.

627. Diptera (Henking, *Zeit. wiss. Zool.*, xlvi, 1888, p. 289).—Ova still contained within the fly may be fixed by plunging the animal for some time into boiling water, then dissecting out and bringing them into 70 per cent. alcohol. Laid eggs may have boiling water poured over them, or be put into solution of Flemming in a test-tube which is plunged into boiling water until the eggs begin to darken (about a minute). Cold solution of Flemming easily causes a certain vacuolisation of the contents of the ova. Open the ova at the larger end, stain with borax-carmine for fifteen to thirty hours, and cut in paraffin.

Bruel (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 569) fixes larvæ and pupæ in absolute alcohol heated to 70° to 75° C., and containing "a little" sublimate. See also Van Rees, *ibid.*, iii, 1888, p. 10.

Bengtsson (*Handl. Fysiogr. Sællsk Lund.*, viii, 1897) finds hot alcoholic solution of sublimate (Frenzel's, § 69) the best fixative for larvæ of *Phalacrocera*. He could not succeed in softening the chitin with *eau de Javelle*.

Pérez (*Arch. Zool. expér.*, (4), v, 1910, p. 11) fixes pupæ in Bouin's picro-formol, or Marchoux's mixture, for twenty-four hours.

628. Lepidoptera (Bobretzky, *Zeit. wiss. Zool.*, 1879, p. 198).—Ova are slightly warmed in water and put for sixteen to twenty hours in 0·5 per cent. chromic acid. The membranes can then be removed.

629. Hymenoptera.—Carrière & Bürger (*Nova Acta Acad. Leop. Car.*, lxix, 1897, p. 273) kill ova of *Chalicodoma* by warming in water to 60° C., and fix in aqueous picric acid, or alcohol of 70 per cent.

Petrunkewitsch (*Zool. Jahrb. Abth. Morph.*, xiv, 1901, p. 576) fixes for twenty-four hours in his sublimate mixture, and passes into alcohol of 70 per cent. with iodine.

630. Orthoptera (Patten, *Quart. Journ. Mic. Sci.*, 1884, p. 549).—The ova or larvæ (of Blattida) are placed in cold water, which is gradually raised to 80° C. You leave off heating as soon as the ova have become hard and white. Pass very gradually through successive alcohols, beginning with 20 per cent.

Wheeler (*Journ. of Morph.*, iii, 1889, p. 292) dissects out ovarian ova in salt solution and fixes in liquid of Perényi (fifteen minutes), then treats with alcohol, and stains with borax-carmine. Laid eggs may be killed by Patten's method. After heating, the two lips of the crista of the capsule may be separated with fine forceps and pieces of the walls torn away, and the eggs pushed out of the compartments formed by their choria and hardened as desired. Good results are also obtained by heating to 80° C. for ten minutes in liquid of Kleinenberg, and preserving in 70 per cent. alcohol. This causes the envelopes to dilate and stand off from the surface of the egg, so that they can easily be dissected away.

Heymons (*Zeit. wiss. Zool.*, liii, 1892, p. 434), for young embryos, incises the cocoon at the end by which it adheres in the body of the mother, brings it for two minutes into water heated to 90° C., and opens in Flemming, in which the embryo is dissected out.

Morgan (*Amer. Natural.*, xxii, 1888, p. 357) puts ova of *Periplaneta* for thirty minutes or an hour into eau de Javelle diluted with 4 to 8 vols. of water and slightly warmed, which softens the capsules.

631. Coleoptera.—Hirschler (*Zeit. wiss. Zool.*, xcii, 1909, p. 628) fixes ova of *Donacia* (after incising the chorion) for two to three hours in equal parts of sublimate of 6 per cent. and nitric acid of 3 per cent.

Saling (*Dissert. Marburg*, 1906, p. 10) fixes ova of *Tenebrio* for about two minutes in a hot mixture of 40 parts of alcohol of 96 per cent., 4 of nitric acid, and 50 of saturated aqueous sublimate; or for 3 minutes in a hot mixture of 1 part of formol with 3 of water.

Karawaiew (*Biol. Centralb.*, xix, 1899, p. 124) kills larvæ of *Anobium* in hot water, freezes them with ether spray, cuts away a lateral strip, lets them thaw, and puts for 24 hours into picro-sulphuric acid.

632. Phalangida.—The ova of *Phalangium opilio* possess a chorion covered with yellow corpuscles that render them opaque. Balbiani puts them into water with a few drops of caustic potash, and raises to boiling point. The ova are then laid on filter paper, and the chorion removed by rubbing with a camel's hair brush, the vitelline membrane remaining intact, so that the embryo can be studied through it.

Henking's method (*Zeit. wiss. Zool.*, xlv, 1886, p. 86).—Fix with boiling water or Flemming. Preserve the ova in 90 per cent. alcohol. To open the chorion, bring them back into 70 per cent. alcohol, which causes them to swell up so that the chorion can easily be pierced with needles, and the ovum turned out.

633. Araneida.—Kishinouye (*Journ. Coll. Sci. Imp. Univ. Japan*, iv, 1891, p. 55; *Zeit. wiss. Mik.*, ix, 1892, p. 215) fixes in water warmed to 70° or 80° C., puts into 70 per cent. alcohol, and after twenty-four hours therein pierces the membranes and passes through stronger alcohol.

See also Locy, *Bull. Mus. Comp. Zool. Harvard*, xii, 3, 1886. Fix by hot water. The liquid of Perényi may also be used; it has the advantage of not making the yolk so granular.

Montgomery (*Journ. Morph.*, xx, 1909, p. 628) fixes ova of *Theridium* for 1 or 2 hours in Carnoy & Lebrun's mixture.

Lambert (*ibid.*, p. 420) fixes ova of *Epeira* in picro-sulphuric acid warmed to 70° or 80° C.

Purcell (*Quart. Journ. Micr. Sci.*, liv, 1909, p. 7) fixes ova of *Atta* in boiling saturated sol. of sublimate in alcohol of 70 per cent.

HAMBURGER (*Zeit. wiss. Zool.*, xcvi, 1910, p. 3) fixes ova of *Argyroneta* in Gilson's mixture.

634. **Limulus.**—KINGSLEY (*Journ. Morph.*, vii, 1892, p. 38) kills ova by heating in sea-water to 70° or 75° C. and brings into alcohol of 30 to 70 per cent. Similarly KISHINOUYE, *Journ. Coll. Sci. Japan*, v, 1893, p. 56.

635. **Decapoda.**—REICHENBACH (*Abh. Senckenberg Ges. Frankfurt*, xiv, 1886, p. 2) fixes ova of *Astacus* in water gradually warmed to 60° or 70° C. (if the chorion should burst, that is no evil), hardens for twenty-four hours in 1 to 2 per cent. bichromate of potash or 0·5 per cent. chromic acid, washes out for the same time in running water, and brings into alcohol. Remove the chorion, and remove the embryo from the yolk with a sharp knife.

HERRICK (*Bull. U. S. Fish. Comm.*, xv, 1896, p. 226) kills the ova in hot water, shells and fixes in picro-sulphuric acid.

For *Homarus*, see WAITE, *Bull. Mus. Comp. Zool.*, xxxv, 1899, p. 155.

636. **Amphipoda.**—DELLA VALLE (*Fauna u. Flora Golf. Neapel*, xx, Monog., 1893, p. 170) puts ova of *Orchestia* by means of a pipette into boiling, cold-saturated sublimate solution, removes them instantly into sea-water, and thence into weak alcohol. If the chorion does not burst of itself it must be pricked with a needle.

637. **Cladocera.**—HAEKER (*Zellen. u. Befruchtungslehre*, 1899, p. 60) fixes females of *Sida* with winter eggs in a hot mixture of 100 c.c. alcohol of 70 per cent. with 1 to 2 c.c. saturated sol. of sublimate. See also SAMTER, *Zeit. wiss. Zool.*, lxviii, 1900, p. 176.

638. **Copepoda.**—KRUEGER (*Arch. Zellforsch.*, vi, 1911, p. 173) fixes ovaries of Harpactida in Zenker's mixture with 10 per cent. of formol added. No other liquids give good results.

Vermes.

639. Rotatoria.—Jennings (*Bull. Mus. Harvard Coll.*, xxx, 1896, p. 101) finds the best fixative for pregnant females is the strong liquid of Flemming, but the ova must then be bleached with chlorate of potash (§ 575).

Lenssen (*La Cellule*, xiv, 1898, p. 428) fixes ova of *Hydatina* with sublimate for 20 seconds.

640. Turbellaria.—Gardiner (*Journ. of Morph.*, xi, 1895, p. 158) finds the best fixative for ova of *Polychoerus* is a mixture of equal parts of absolute alcohol and glacial acetic acid.

Bresslau (*Zeit. wiss. Zool.*, lxxvi, 1904, p. 219) fixes Mesostomidæ with summer-eggs in Tellyesniczky's mixture (either cold or warmed to 60° or 70° C.) for 10 to 12 hours, and washes out for the same time. He incises winter-ova at one pole, fixes and brings into alcohol of 95 per cent., then makes an incision at the other pole, and imbeds in paraffin through cedar oil. In the paraffin, slices of the shell may be removed with a scalpel, and the ova re-imbedded when sufficiently shelled.

Van der Stricht (*Arch. Biol.*, xv, 1898, p. 370) finds that ova of *Thysanozoon* will only cut well when they have been not more than two minutes in absolute alcohol followed by chloroform and paraffin as used by Carnoy and Lebrun, § 603.

See also, for Polyclads, Francotte, *Arch. Zool. Expér.*, vi, 1898, p. 196; and, for fresh-water Planaria, Iijima, *Zeit. wiss. Zool.*, xl, 1884, p. 359.

641. Cestoda (v. Beneden, *Arch. Biol.*, ii, 1881, p. 187).—Ova of *Tænia* in which a chitinous membrane has formed around the embryo are impervious to reagents. They may be put on a slide with a drop of some liquid and covered. Then, by withdrawing the liquid by means of blotting-paper, the cover may be made to gradually press on them so as to burst the membranes, and the embryo may then be treated with the usual reagents.

Haswell (*Quart. Journ. Micr. Sci.*, liv, 1909, p. 417) fixes ova of Temnocephala in "sublimate alcohol," brings them into 90 per cent. alcohol with iodine added, and thence gradually back into water, softens the shells in weak sodium hypochlorite, washes and imbeds.

642. **Trematoda.**—Coe (*Zool. Jahrb., Abth. Morph.*, ix, 1896, pp. 563, 566), for the special study of the excretory system of the Miracidia of *Distomum*, kills with osmic acid, rinses with distilled water, and puts for a couple of days into ¼ per cent. solution of silver nitrate.

Egg-capsules may be softened with 5 per cent. caustic potash and then burst open (Heckert, *Bibl. Zool.*, iv, 1889).

643. **Nematoda.**—The ova of *Ascaris megalocephala*, a classical object of study, are one of the most impervious things in the animal kingdom. Years ago Fol related to me that he had had ova segmenting right through absolute alcohol into balsam. Bataillon (*Arch. Entwickelungsmech.*, 1901, p. 149) has had ova showing living embryos after having been for six months in liquid of Flemming, and found them to remain alive for months after drying for 24 hours at 35° C., and mounting in balsam, and for weeks in acids or alkalies.

Doubtless the best fixative yet made known for ova furnished with their capsules will be found to be that of Carnoy and Lebrun, § 86 (*La Cellule*, xiii, 1897, p. 68). After fixation the ova are carefully brought into 80 per cent. alcohol, in which they are preserved. Imbedding should be carefully done as recommended for the ova of Amphibia (§ 603), but they ought not to remain in the pure paraffin for more than a minute to a minute and a half. But these authors prefer the celloidin method. At least six weeks' soaking in the different strengths of celloidin will be necessary to ensure penetration. They stain with iron hæmatoxylin.

Zur Strassen (*Arch. Entwickelungsmech.*, iii, 1896, p. 29) fixes for twenty-four hours in a mixture of 4 parts 96 per cent. alcohol and 1 part acetic acid, brings into pure alcohol, stains with hydrochloric acid carmine, and brings gradually into glycerin.

Similarly Zoja (*Arch. mik. Anat.*, xlvii, 1896, p. 218) and Erlanger (*ibid.*, xlix, 1897, p. 309). Zoja stained with Bismarck brown and examined in dilute glycerin; Erlanger made paraffin sections and stained with iron hæmatoxylin.

Kostanecki and Siedlecki (*ibid.*, xlviii, 1896, p. 184) employed concentrated sublimate solution, or 3 per cent. nitric acid or mixtures of these two, for ovarian ova.

Van Beneden and Neyt (*Bull. Acad. Belg.*, 1887, p. 214) took equal parts of alcohol and acetic acid. Boveri (*Jena Zeit.*, xxi, 1887, p. 423) fixed in his picro-acetic acid, § 95—a clearly inadequate method. Gulick (*Arch. Zellforsch.*, vi, 1911) has "fixed" ova of *Heterakis* for 22 hours in one third saturated picric acid with 3 per cent. of glacial acetic acid, and had them develop in alcohol of 70 per cent. to stages representing a normal development of several weeks.

Boring (*Arch. Zellforsch.*, iv, 1909, p. 121) spreads ova of *Ascaris* on a layer of Mayer's albumen on a slide, sets the albumen with a drop of formol, fixes with 4 parts of alcohol to 1 of acetic acid, stains in alcoholic hydrochloric acid carmine, and mounts in glycerin.

Artom (*Zeit. wiss. Mik.*, xxv, 1908, p. 5) freezes segments of the uteri of *Ascaris* in salt water, and cuts them with the freezing microtome into disks 30 μ thick, and fixes these with divers liquids.

Cerfontaine (*ibid.*, xxix, 1912, p. 305) brings fixed ova from alcohol into absolute alcohol with 1 per cent. of clove oil, evaporates this down to one tenth, puts into absolute alcohol with 5 per cent. of clove oil, evaporates again down to one tenth, then into the same with 5 per cent. of collodion added, evaporates almost entirely away, and passes through cedar oil into paraffin.

Echinodermata, Cœlenterata, and Porifera.

See the chapter on "Zoological Methods."

CHAPTER XXVI.

CYTOLOGICAL METHODS.

644. Study of Living Cells.—In the young larvæ of Amphibia, both Anura and Urodela, the gills and caudal "fin," and sometimes other regions, may be studied in the living state.

The larvæ may be fixed in a suitable cell, or wrapped in moist blotting-paper, or may be curarised; or the tail may be excised. (It is preferable to cut through the larva close in *front* of the hind limbs.)

In the *living animal* the epithelial cells and nuclei (in the state of repose) are so transparent as to be hardly visible in the natural state. They may, however, be brought out by curarising the larva; or, still better, by placing the curarised larva for half an hour in 1 per cent. chloride of sodium solution. Normal larvæ may be used for the study of the active state of the nucleus, but much time is saved by using curare.

Curare.—Dissolve 1 part of curare in 100 parts water, and add 100 parts of glycerin. Of this mixture add from 5 to 10 drops (according to the size of the larva), or even more for large larvæ, to a watch-glassful of water. From half to one hour of immersion is necessary for curarisation. The larvæ need not be left in the solution until they become quite motionless; as soon as their movements have become slow they may be taken out and placed on a slide, wrapped in blotting-paper. If they be replaced in water they return to the normal state in eight or ten hours, and may be re-curarised several times.

Other Narcotics.—Three per cent. alcohol or 3 per cent. ether, or infusion of tobacco, may be used in a similar way.

These reagents cause no obstruction to the processes of cell-division.

Indifferent Media.—One per cent. salt solution, iodised serum, syrup, cold water (+ 1° C.), and warm water (35°—40° C.). The tail may be excised from the living animal and studied for a long time in these media (Peremeschko, *Arch. mik. Anat.*, xvi, 1879, p. 437).

For the processes of *staining* living cells see § 208.

645. **Study of Fresh and Lightly Fixed Cells.**—So-called "indifferent" liquids must not be believed to be without action on nuclei. Iodised serum, salt solution, serum, aqueous humour, lymph, better deserve the name of weak hardening agents. Between these and such energetic hardening agents as Flemming's mixture come such light fixing agents as picric acid or very dilute acetic acid. These it is whose employment is indicated for the study of fresh isolated cells.

A typical example of this kind of work is as follows: Tease out a piece of living tissue in a drop of acidulated solution of methyl green (0·75 per cent. of acetic acid). This is a delicate fixing agent, killing cells instantly without change of form. Complete the fixation by exposing the preparation for a quarter of an hour to vapour of osmium, and add a drop of solution of Ripart and Petit and a cover.

Or you may fix the preparation, after teasing, with vapour of osmium for half a minute to two minutes, then add a drop of methyl green, and after five minutes wash out with 1 per cent. acetic acid, and add solution of Ripart and Petit and cover.

Or you may kill and fix the cells by teasing in solution of Ripart and Petit (to which you may add a trace of osmic acid if you like), and afterwards stain with methyl green.

I have found Pictet's chloride of manganese (§ 403) useful as an examination medium. A little solution of dahlia may be added to it.

Henking (*Zeit. wiss. Mik.*, viii, 1891, p. 156) recommends a liquid composed of—

Water	80 c.c.
Glycerin	16 ,,
Formic acid	3 ,,
Osmic acid of 1 per cent. . . .	1 ,,
Dahlia	0·04 grm.

Other fixing agents, such as picric acid or weak sublimate solution, may of course be used. Other stains, too, such as Bismarck brown, and of course other examination media than solution of Ripart may be employed. But, for general purposes, the methyl-green-osmium-and-Ripart's-medium method gives such good results, and is so very convenient, that it may be called a classical method for the study of fresh cells.

646. Some Microchemical Reactions.—*Methyl green* is a test for *chromatin*, in so far as (with *fresh* cells) it colours nothing but the chromatin *in the nucleus*, *see* § 276. It is, however, not a perfect test, for the intensity of the coloration it produces varies greatly in different nuclei, and may in certain nuclei be extremely weak, or (apparently) even altogether wanting. In these cases other tests must be applied in order to establish with certainty the presence or absence of that element.

Chromatin is distinguished from albuminoids by not being soluble, as these are, in water and in weak mineral acids, such as 0·1 per cent. hydrochloric acid. It is easily soluble in concentrated mineral acids, in alkalies, even when very dilute, and in some alkaline salts, such as carbonate of potash and biphosphate of soda. In the presence of 10 per cent. solution of sodium chloride it swells up into a gelatinous mass, or even, as frequently happens, dissolves entirely (Carnoy, *Biol. Cell.*, pp. 208—9). It is only partially digestible (when *in situ* in the nucleus) in the usual laboratory digestion fluids.

The solvents of chromatin that are the most useful in practice are 1 per cent. caustic potash, fuming hydrochloric acid, or cyanide of potassium, or carbonate of potash. These last generally give better results than dilute alkalies. They may be employed in solutions of 40 to 50 per cent. strength. If it be desired to remove all the chromatin from a nucleus the reaction must be prolonged, sometimes to as much as two or three days, especially if the operation be conducted on a slide and under a cover-glass, which is the safer plan.

These operations must be performed on *fresh* cells, for hardening agents render chromatin almost insoluble in ammonia, potash, or sodic phosphate, etc. Hydrochloric acid, however, still swells and dissolves it, though with difficulty.

Chromatin resists the action of *digestive fluids* much longer than the albumins do; so that a moderate digestion serves to free the chromosomes from any caryoplasmic granulations that may obscure them, whilst at the same time it clears up the cytoplasm. UNNA (*Monatschr. prakt. Derm.*, xxxiii, 1901, p. 342) digests tissues in solutions of sodium chloride, to remove the granoplasm.

For *Glycogen* see CREIGHTON, *The Formative Property of Glycogen*, London, 1896; GAGE, *Trans. Amer. Micr. Soc.*, xxviii, 1908, p. 203; KATO, *Arch. Ges. Phys.*, cxxvii, 1909, p. 125; BEST, *Zeit. wiss. Mikr.*, xx, 1904, p. 358, and xxiii, 1909, p. 319; BUSCH, *Arch. Intern. Phys.*, iii, 1905, p. 51; MAYER, *Zeit. wiss. Mikr.*, xxvi, 1909, p. 513; ARNOLD, *Sitzb. Heidelberg. Acad. Wiss.*, 1909, p. 1, 1910, p. 3, and 1911, 14 Abh.; *Arch. Path. Anat.*, cxciii, 1908, p. 175; *Arch. mik. Anat.*, lxxiii, 1909, p. 265; lxxvii, 1911, p. 346; *Beitr. path. Anat.*, li, 1911, p. 439; FRAENKEL, *Virchow's Arch.*, 1911, p. 197; ZIEGWALLNER, *Zeit. wiss. Mik.*, xxviii, 1911, p. 152; NEUBERT, *Beitr. path. Anat.*, xlv, 1909, p. 38; ERHARD, *Arch. Zellforsch.*, viii, 1912, pp. 447 and 507; EHRLICH and LAZARUS, *Die Anaemie*, 1898, p. 30; PEKELHARING, *Petrus Camper*, Deel I, 1901, p. 231; DRIESSEN, *Zeit. wiss. Mik.*, xxii, 1905, p. 422; FISCHER, *Anat. Anz.*, xxvi, 1905, p. 399; FIESSINGER, *C. R. Soc. Biol.*, lxvi, 1909, p. 183; NEUKIRCH, *Arch. path. Anat.*, cc, 1910, p. 82; VASTARINI-CRESI, *Att. Acc. Med. Chir. Napoli*, xli, 1907, p. 350, and xliii, 1909, p. 109; SILBERMANN and OZOROWITZ, *Bull. Soc. Sci. Bucarest*, xvii, 1908, p. 43.

For *Phosphorus* see MACALLUM, *Proc. Roy. Soc.*, lxiii, 1898, p. 467; HEINE, *Zeit. Phys. Chemie*, xxii, 1896, p. 132; BENSLEY, *Biol. Bull. Wood's Holl*, x, 1906, p. 62; SCOTT, *Journ. Phys. Cambridge*, xxxv, 1906, p. 119.

For *Iron* see MACALLUM, *Ergeb. Phys. Wiesbaden*, vii, 1908, p. 565; TIRMANN, *Goerbersdorfer Veroeffentl.*, ii, 1898, p. 111; MACALLUM, *Quart. Journ. Micr. Sci.*, xxxviii, 1895, p. 175; SCHNEIDER, *Mitth. Zool. Stat. Neapel*, xii, 1895, p. 208; CARNOY and LEBRUN, *La Cellule*, xii, 1897, p. 275; SUMITA, *Arch. path. Anat.*, cc, 1910, p. 220; ZALESKI, *Zeit. Phys. Chemie*, xiv, 1890; WASSERMANN, *Anat. Hefte*, xlii, 1910, p. 283.

For *Copper* see BOYCE and HERDMAN, *Proc. Roy. Soc.*, lxii, 1897, p. 35; MACALLUM, *Journ. Phys. Cambridge*, xxii, 1897, p. 92; MARFORI, *Arch. Ital. Biol.*, xxx, 1898, p. 186.

For *Zinc* see MENDEL and BRADLEY, *Amer. Journ. Phys.*, xiv, 1905, p. 320.

For *Lime salts* see GRANDIS and MAININI, *Arch. Ital. Biol.*, xxxiv, 1900, p. 75; SCHAFFER, *Zeit. wiss. Zool.*, lxxxix, 1908, p. 13; LEUTERT, *Encycl. mikr. Technik*, ii, p. 588; STOELTZNER, *Arch. path. Anat.*, clxxx, 1905, p. 363 MACALLUM, *Ergeb. Phys. Wiesbaden*, vii, 1908, p. 612.

For *Potassium* see MACALLUM, *Journ. Phys. Cambridge*, xxxii, 1905, p. 95; *Ergeb. Phys. Wiesbaden*, vii, 1908, p. 600.

For *Guanin* see GIACOMO, *Zeit. wiss. Mik.*, xxvii, 1910, p. 257.

Concerning the microchemistry of the cell in general see further *fourth edition;* also CARNOY & LEBRUN, *La Cellule*, xii, 2, 1897, p. 194; ZIMMERMANN, *Die Morphologie u. Physiologie des Pflanzlichen Zellkernes*, Jena, 1896 (treats also of the animal cell); HAECKER, *Praxis u. Theorie der Zellenund Befruchtungslehre*, Jena; PRENANT, *Journ. Anat. Phys.*, xlvi, 1910, p. 343.

647. Cytological Fixing Agents.—A fixing agent that is good for one element of a cell is not necessarily good for all others.

As regards the nucleus, all fixatives should be *acid;* for if not they will not satisfactorily preserve either chromatin or nucleoli.

For instance, bichromate of potash, if not rendered acid, fixes chromosomes and nucleoli in a distended state so that clear images of them are not obtained. Acids contract them somewhat, and so give them sharper outlines.

The fixatives mostly employed for nuclei are liquid of FLEMMING and liquid of HERMANN. There is a slight difference between them. Liquid of Hermann, owing to the platinum chloride, causes chromatin to shrink more than liquid of Flemming does, and for this reason is supposed to give clearer images of chromosomes, especially of their splitting. I find that it generally make sthem shrink *too much,* and that it is not at all good for spindles.

For many, if not most objects, I prefer to these two reagents BOUIN's picro-formol, which gives a highly faithful preservation and a *more penetrating and equable* fixation.

Alongside of this I would place CARNOY & LEBRUN's acetic-acid-sublimate fluid, which gives equally fine images of chromosomes, and is still more penetrating. It is, however, not so good for spindles. *For these* I recommend Flemming (picro-formol does not give quite such bold images).

Some of the finest chromosomes I have seen have been fixed with LINDSAY JOHNSON's mixture (§ 44), and liquid of TELLYESNICZKY has given me others nearly if not quite as good.

As regards the cytoplasm. Cytoplasm is made up of two elements: a fibrillar element—the spongioplasm or mitome;

and a more or less granular liquid that bathes it—the hyaloplasm or enchylema. It does not follow that a reagent that will fix one of these will also fix the other. Nor is it always desirable that both should be equally fixed.

If you fix both, you will have a *full* fixation; but in that case the granules of the hyaloplasm (be they vital, or be they only "precipitation forms," see § 29), and the secretions or other enclosures that may be present in it, may so mask the fibrils of the spongioplasm as to interfere with the observation of it. So that if the latter is the principal object of study, a *thin* fixation, one in which the spongioplasm is entirely preserved, but the hyaloplasm only partly, may be the better.

The *spongioplasm* is the easier to fix of the two, and the majority of acid fixatives will preserve it more or less. The best images I have obtained are those given by liquid of Flemming or Hermann in cells in which the action of the reagent has been moderate, *i. e.* insufficient to thoroughly fix the hyaloplasm at the same time. Nearly, if not quite, as good, is Bouin's picro-formol, which has the great advantage of being very favourable for plasma-staining. I have also had very good results with vom Rath's picro-osmic and picro-platinosmic mixtures, and with acid sublimate.

Hyaloplasm is not nearly so easy to fix, and there are only two reagents in common use that readily give a really full fixation of it; these are *osmic acid* and *bichromate of potash*.

Osmic acid acts as a fixative of hyaloplasm in liquid of Flemming or Hermann, but only gives a full fixation in the outer layers of the material; and in these it easily happens that many or most of the cells are ruined by over-fixation (see § 35).

This defect may be to a certain degree corrected by taking the osmic acid weaker than is usual. Thus by successively reducing the proportion of this ingredient in liquid of Hermann,* I have found that it can be brought down to

---

* NIESSING (*Arch. mik. Anat.*, xlvi, 1895, p. 147) has the following two modifications of Hermann's mixture:

(1)	Platinum chloride, 10 per cent. solution	25
	Osmic acid, 2 per cent.	20
	Glacial acetic acid	5
	Distilled water	50

one eighth of the prescribed amount without loss of the distinctive characters of the fixation.

The defect of want of penetration seems to be incurable (see §§ 35 and 42). Substitution of more highly penetrating reagents, such as picric acid, for the chromic acid or platinum chloride, does not help in the least; you only get the osmic fixation outside, no whit deeper than before, and a picro-acetic fixation, instead of a chromo- or platino-acetic one, in the deeper layers, that is all.

In view of these defects of osmic mixtures, it may often be advisable, where hyaloplasm, or its enclosures, is the chief object of study, to have recourse to *bichromate of potash*. The formula that has given me the finest fixations is that of LINDSAY JOHNSON, but it has the drawback that there is risk of osmication in the outer layers.

In this respect liquid of Tellyesniczky, § 52, is to be preferred.

Corrosive sublimate gives a fairly full fixation; but I believe it frequently produces serious artefacts, HEIDENHAIN'S "Lanthanin" being one of them. Heidenhain's solution, § 64, containing as it does some 11 per cent. of sublimate, without the addition of any acid to neutralise its shrinking action, seems to me to be an inadmissibly coarse reagent. I have, however, obtained with liquid of Carnoy-Lebrun, § 86, some most excellent fixations of cytoplasm. The aqueous solutions of sublimate are frequently used in preference to liquid of Flemming on account of the facilities they afford for the employment of certain stains; but to that end I prefer BOUIN'S picro-formol.

ALTMANN'S fixtives for nuclei see *fifth edition*, or *Arch. Anat. Entw.*, 1892, p. 223, and his *Elementarorganismen*, 1890. His mixture for his granula see § 43. See also THÉOHARI (*Journ. de l'Anat.*, xxxvi, 1900, p. 216).

---

(2) The same with a saturated aqueous solution of corrosive sublimate instead of the water.

They are both of them, in my opinion, as ill-imagined as possible. They contain some three times as much platinum chloride as Hermann's, and Hermann's contains already quite as much as it can bear, and, I think, much more than is advisable: see the proportions in the mixtures §§ 44 and 49. RABL (*Anat. Anz.*, iv, 1889, p. 21) employed it of from $\frac{1}{10}$ to $\frac{1}{8}$ per cent. strength, which seems to me much nearer the mark.

648. Chromatin Stains.—For fresh tissues see § 645.

For sections of hardened tissues, stains should be chosen amongst those that give a very intense as well as a very sharp coloration. Some years ago safranin and gentian violet were the most used. At the present time their place has been taken by the iron hæmatoxylin of Benda or Heidenhain. An alum hæmatoxylin such as *well-ripened* Delafield's, or Ehrlich's, or hæmalum, may also give very good results.

See also Thionin, Kernschwarz, and Iron Carmine, § 220. For Bataillon and Koehler's borax-methylen-blue see *Comptes Rendus*, cxvii, 1893, p. 521.

649. Nucleoli are "acidophilous" in so far as, in fixed material, they select the acid dye or dyes from mixtures such as the Ehrlich-Biondi stain. With this they stain mostly red, sometimes orange. With *fresh* material they do not stain at all with acid methyl green (distinction from chromatin).

But in fixed material treated with basic dyes (safranin, gentian, etc.) by the regressive method they stain more energetically than resting chromatin, and at least as much so as chromatin in the kinetic state. With iron-hæmatoxylin they stain sometimes full black, sometimes grey with a black shell.

They can frequently be well demonstrated in unstained preparations examined in water, being brought out by their superior refractivity, and are sometimes visible in the living cell.

One of the best ways of demonstrating them is to fix with strong liquid of Flemming, and stain with safranin, followed by differentiation with acid alcohol (§ 286).

Obst (*Zeit. wiss. Zool.*, lxvi, 1899) fixes in sublimate, stains in borax carmine, and then stains the sections for three hours in very dilute aqueous methyl green or solid green. Nucleoli blue, chromatin red.

Buchner (*Arch. Zellforsch.*, iii, 1909, p. 337) has found this useful for distinguishing the accessory chromosome in testis cells of Orthoptera—normal chromosomes red, accessory and chromatin nucleoli blue-violet.

Zimmermann (*Zeit. wiss. Mik.*, 1896, p. 463) stains for 10 minutes in a fresh mixture of 9 parts 0·1 per cent. aqueous iodine green with 11 parts concentrated aqueous solution of fuchsin, and differentiates in absolute alcohol with 1 per cent. of acetic acid and 0·1 per cent. of iodine. Nucleoli red, chromatin blue.

Fischer (*Fixirung*, etc., p. 140) adds 30 drops of hot 0·1 per cent. fuchsin solution to 100 c.c. of 0·3 to 0·5 per cent. solution of methyl green.

Montgomery (*Journ. Morph.*, xv, 1899) stains for an hour in Ehrlich's hæmatoxylin, and then for five minutes in concentrated aqueous eosin, or first with concentrated aqueous methylen blue, and then with concentrated alcoholic solution of Brazilin.

See also Reddingius, *Virchow's Arch.*, clxii, 1900, p. 206. For nucleoli of ova, List, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 480; of nerve-cells, Ruzicka, *Zeit. wiss. Mik.*, xiv, 1898, p. 453, and Levi, *Riv. Pat. Nerv. Ment. Firenze*, iii, 1898, p. 289.

650. Plasma Stains.—I have been unable to discover a single thoroughly satisfactory one. Almost all of them colour too readily the enchylema or hyaloplasm at the same time as the spongioplasm. And, on the other hand, there are many important elements of cells which cannot be got to stain sufficiently.

I consider Säurefuchsin the most generally recommendable, especially after iron hæmatoxylin. See also Bordeaux R.

Flemming's Orange Method has been much used. It is very capricious and unreliable.

Ehrlich-Biondi mixture is a celebrated plasma stain.

The *Iron-Hæmatein Lakes* of Benda and M. Heidenhain give good plasma stains, according to the degree of extraction, and would be inferior to none were it not that they stain in the same tone as the chromatin. See also Ehrlich's *tri-acid*, and his *acidophilous mixture*, also *gold chloride*, Apáthy's process, § 371, and *Kernschwarz*.

Imperfectly stained plasma structures can often be well brought out by mounting in *Euparal* instead of balsam.

651. Centrosomes.—These *can* be stained by some "acid" anilin dyes, better by a "neutral" dye (*e. g.* Flemming's orange method, or the Ehrlich-Biondi-Heidenhain stain). But *by far the best* stain is *iron-hæmatoxylin.*

It is said by Heidenhain that the stain is obtained in a sharper form by combining the hæmatoxylin stain with a foregoing stain with *Bordeaux R.* He directs (*Arch. mik. Anat.*, xlii, 1894, p. 665) that the sections (sublimate sections were used by him) are to be stained for twenty-four hours or more in "a weak" solution of Bordeaux, until they have attained such an intensity of colour as that "they would just be fit for microscopic examination with high powers" (*l. c.*, p. 440, note), and that they be then brought into the ferric alum. After mordanting and staining, the hæmatoxylin is to be extracted in the iron alum until the chromatin has become entirely or almost entirely colourless. Instead of Bordeaux, "anilin blue" may be used in the same way.

The images of these objects given by iron-hæmatoxylin require to be interpreted with special care. For they sometimes exhibit the phenomenon termed by Fischer (*Fixirung, Färbung und Bau des Protoplasmas*, 1899, p. 31, *et passim*) "Spiegelfaerbung," that is—a *bull's eye effect.* Globular or even elongated objects, such as chromosomes, do not always yield up their stain simultaneously and equally throughout their whole depth, but lose it suddenly and entirely in their outer layers, whilst retaining it in its full strength in their deeper layers. The still-stained parts thus remain separated from the decoloured parts by a sharply-defined limit; so that a spherical granule in this state will show a central point deeply stained—the bull's eye—and around it a perfectly colourless area—the white of the target. And when the object is in balsam it is frequently quite impossible to distinguish the outer limit of this colourless area, so that the whole object appears to have only the dimensions of the stained area. It seems that certain erroneous observations that have been published have been due to this deception.

Hermann (*Arch. mik. Anat.*, xxxvii, 4, 1891, p. 583) recommends a modification of the hæmatoxylin impregnation method of Pal, for which see *fourth edition;* also his paper, "Methoden zum Studium des Archoplasmas" in *Ergebnisse der Anatomie*, Band ii, 1892 (1893), p. 23.

For Heidenhain's *Vanadium hæmatoxylin*, see § 269.

Benda (*Verh. Phys. Ges. Berlin*, November, 1900, Nr. 1–2; *Verh. Anat. Ges.*, xv, 1901, p. 167) gives the following as succedanea of the iron hæmatoxylin method: The material is to be fixed in alcohol of about 93 per cent. for two days, then treated for twenty-four hours with nitric acid diluted with 10 vols. of water. Then bichromate of potash of 2 per cent., twenty-four hours; chromic acid of 1 per cent., forty-eight hours; water, twenty-four hours; alcohol; paraffin (or sections by freezing). The sections are stained either by *iron hæmatoxylin* (twenty-four hours in the mordant and in the stain, with differentiation in Weigert's borax-ferricyanide), or by either of the two following methods:

(*a*) The sections are oxidised for five minutes in 0·5 per cent. solution of permanganate of potash, reduced in Pal's oxalic mixture till they become white (about three minutes), dried with blotting-paper flooded with Weigert's methyl-violet-oxalic mixture, or with the crystal-violet solution, § 330, dried, rinsed with solution of Lugol, rinsed, dried again with blotting paper, differentiated with a mixture of equal parts of xylol and anilin oil, dried, rinsed with xylol, balsam.

(*b*) Sections mordanted twenty-four hours in iron alum of 4 per cent. or *liquor ferri*, § 241, diluted with 2 vols. of water, rinsed, stained twenty-four hours in the sol. of sulphalizarinate of soda, § 653, rinsed, mopped with blotting-paper, warmed in 0·1 per cent. sol. of toluidin blue till vapour is given off, stained fifteen minutes more in the same solution whilst cooling, dipped in acetic acid of 1 per cent., dried with blotting-paper, dipped in alcohol, differentiated about ten minutes in beech-wood creosote, dried with blotting-paper, rinsed many times with xylol, then balsam.

The *Nebenkern* of spermatic cells may be studied by the methods indicated for centrosomes. *Kernschwarz* is also very useful here.

652. Cell Granules.—For the study of the conspicuous "granules," undoubtedly metabolic products, occurring in certain gland-cells and blood- and lymph-corpuscles, and in certain elements belonging to the group of connective tissues, see the sections on "Connective Tissues." The most generally employed stains are the mixtures of Ehrlich.

Intra vitam staining is useful here (see § 208). See also Arnold, *Anat. Anz.*, xxi, 1902, p. 417.

Benda (*Verh. phys. Ges. Berlin*, 1899–1900, Nr. 1–4, and *Verh. Anat. Ges.*, xv, 1901, p. 172) gives the following method for demonstrating *secretion-granules* and distinguishing them from other granules: Harden for 24 hours in 10 per cent. formalin, then for one day in 0·25 per cent. chromic acid, one in 0·33 per cent. and 2 to 3 in 0·5 per cent., wash one day in water, dehydrate and make paraffin sections. Then stain with one of Ehrlich's mixtures, according as the granulations are basophilous, acidophilous, or neutrophilous. The methylen-blue and eosin process of Michaelis is recommended.

For Prenant's *Ergastoplasm* see especially Garnier, *Bibliogr. Anat.*, Nov. 6th, 1897, p. 278, and *Journ. de l'Anat.*, xxxvi, 1900, p. 22, and under "Mitochondria," next §.

ALTMANN (*Studien über die Zelle*, 1886; *Die Elementarorganismen* Leipzig, 1890; *Arch. f. Anat. u. Entwickel.*, 1892, p. 223; also *Zeit. f. wiss. Mik.*, vii. 2, 1890, p. 199; ix, 3, 1893, p. 331; and L. and R. ZOJA, in *Mem. R. Ist. Lombardo di Sci. e Lettere*, xvi, 3, vii, p. 237) demonstrates his "Bioblasts" by fixing for twenty-four hours in a mixture of equal parts of 5 per cent. bichromate of potash and 2 per cent. osmic acid, imbedding in paraffin, staining sections for a minute on the slide held over a flame with a solution of 20 grms. of acid fuchsin in 100 c.c. of anilin water (§ 286), and washing out with saturated alcoholic solution of picric acid diluted with 2 volumes of water, heat being used as before to aid the differentiation, and finally clearing with xylol and mounting in balsam. See hereon the critique of FISCHER, in his *Fixirung, Faerbung, u. Bau des Protoplasmas*, pp. 108, 295 (these granules mainly artefacts).

653. Mitochondria (Chondriosomes, Chondriokonts, Chromidia, Ergastoplasm, etc.).—These formations are fixed, more or less abundantly, by most of the usual fixatives. But some kinds of them seem to be attacked by organic acids; so that it is well to reduce the proportion of these in mixtures. Thus BENDA for this purpose makes up Flemming's strong mixture with only three to six drops of acetic acid to 15 c.c. of the chromic, and 4 c.c. of the osmic. MEVES (*Encycl. mik. Techn.*, 1910, i, p. 476) takes 15 c.c. of chromic acid of 0·5 to 1 per cent., containing 1 per cent. of sodium chloride, with 3 to 4 c.c. of osmic acid of 2 per cent., and three to four drops of acetic acid. Similarly, DUESBERG (*Arch. Zellforsch.*, iv, 1910, p. 605). CHAMPY (*Arch. d'Anat. Mic.*, xiii, 1911, p. 55) takes 7 parts of bichromate of potash of 3 per cent., 7 of chromic acid of 1 per cent., and 4 of osmic acid of 2 per cent. Or iodide of sodium 15 grms.; water 800 grms.; formol 200 grms.; iodide of mercury to saturation. Or simply formol. Some workers take ALTMANN's osmic acid and bichromate, § 43; so MEVES, *Arch. mik. Anat.*, lxxvi, 1911, p. 683.

REGAUD (*Arch. Anat. mic.*, xi, 1910, fasc. 2 and 3) fixes either in 100 parts of 3 per cent. bichromate of potash with 20 of formol and 5 of acetic acid; or 80 parts of the bichromate to 20 of formol *without* acetic acid; or in 20 parts saturated aqueous picric acid with 5 of formol; or in formol of 10 per cent.; and in either case mordants for one to four weeks in bichromate of 3 per cent., and stains in iron hæmatoxylin.

Mitochondria are frequently found stained by many of the current stains, iron hæmatoxylin in particular sometimes staining them with a sharpness that is not attained by any other method. These results are more or less accidental and sporadic: but it is claimed for Benda's alizarin method that it gives a certain and specific stain of them, enabling them to be distinguished from other morphologically similar formations.

Benda's **Alizarin Method** (*Ergebnisse der Anat.*, xii, 1902 (1903), p. 752, and other places) is as follows:—Harden for eight days in strong liquid of Flemming (the acetic acid therein being reduced to three drops). Wash for an hour in water and put for twenty-four hours into a mixture of equal parts of pyroligneous acid and 1 per cent. chromic acid, then for twenty-four hours into bichromate of potash of 2 per cent., wash for twenty-four hours and imbed in paraffin. Sections on the slide are mordanted for twenty-four hours with 4 per cent. solution of ferric alum or diluted *liq. ferri sulfur. oxydat.*, then rinsed with water and put for twenty-four hours into an amber-yellow aqueous solution of Kahlbaum's sulfalizarinate of soda, prepared by dropping 1 c.c. of saturated alcoholic solution thereof into 80 to 100 c.c. of water. Rinse in water, flood the slides with the solution of crystal violet § 330, diluted with an equal vol. of water, and warm till vapour is given off. Rinse, differentiate one or two minutes in 30 per cent. acetic acid (till the nuclei come out reddish), wash in running water for five to ten minutes, dry with blotting paper, dip into absolute alcohol, pass through bergamot oil into xylol and balsam. Mitochondria violet, chromatin and "archoplasm" brown-red, certain secretion granules pale violet, centrosomes red violet.

Instead of the staining solution prescribed above (which may be kept in stock) you may take (*Encycl.*, ii, p. 198) a *freshly prepared* mixture of equal parts of anilin water and saturated alcoholic solution of crystal violet—and this is to be preferred.

Prenant (*Journ. de l'Anat. et Phys.*, xlvi, 1910, p. 217) finds that methylen or toluidin blue, or other basic dyes, may be used instead of the crystal violet.

See also some modifications by Kolster, *Beitr. path. Anat.*, li, 1911, p. 209, consisting in fixation and mordanting in certain chrome alum and chromium fluoride mixtures.

Duesberg (*loc. cit. ante*) has found that the treatment with the chromic and pyroligneous acid and bichromate may be suppressed—with advantage.

Szüts employs the aluminium-alizarin stain given § 335, in lieu of the iron-alizarin for Benda's process.

Some workers (so Meves) prefer to harden as Benda, but to stain with **iron hæmatoxylin** instead of by the alizarin process; the special hardening rendering the hæmatoxylin stain sufficiently specific. Thus also Dingler, *Arch. Zellforsch.*, iv, 1910, p. 673.

Arnold (*ibid.*, viii, 1912, p. 256) stains first with iron hæmatoxylin, differentiates, stains for twenty to thirty minutes with saturated aqueous solution of thionin, passes up to absolute alcohol, stains for two minutes with Orange G. dissolved in clove oil, and passes through xylol into balsam. Chromatin blue, chondriosomes black.

Pensa (*ibid.* p. 612) has studied the mitochondria and chloroplasts in plant cells by Ramón y Cajal's **silver method** for neurofibrils, applied to sections of fresh tissues (a few minutes to an hour in silver of 1 to 2 per cent., reduction for ten minutes to an hour in a hydroquinon bath).

Renaut (*Comptes rend.*, clii, 1911, p. 536) demonstrates mitochondria in fresh cartilage cells by mounting sections in a mixture of artificial serum and saturated aqueous solution of methyl violet 5 B.

CHAPTER XXVII.

TEGUMENTARY ORGANS.

654. Epithelium.—Both for surface views and for sections good results are obtained by the *nitrate of silver* method, the *methylen blue* method, the *perchloride of iron and pyrogallol* method of the Hoggans, § 375, the *osmic acid and pyrogallol* process, § 374, and by *iron-hæmatoxylin.*

For the purpose of separating the epidermis from the corium, Loewy (*Arch. mik. Anat.*, xxxvii, 1891, p. 159) recommends macerating for twenty-four to forty-eight hours, at a temperature of about 40° C., in 6 per cent. pyroligneous acid. Acetic acid of $\frac{1}{3}$ per cent. (Philippson) is also good. Minot (*Amer. Nat.*, xx, 1886, p. 575) macerates embryos for several days in 0·6 per cent. salt solution, Mitrophanow (*Zeit. wiss. Mik.*, v, 1888, p. 573) for a quarter of an hour in 3 per cent. nitric acid, then 1 hour in one-third alcohol, and, if need be, 24 in stronger alcohol.

Mayer (*Lotos*, 2, xii, 1892) exposes the cornea or membrana nictitans of *Rana, Bufo,* and *Mus* for half a minute to the vapour of acetic acid, and then puts it into 0·5 per cent. salt solution.

For *ciliated epithelium* see the methods of Engelmann under "Mollusca."

655. Intercellular Bridges (and Canals), Prickle Cells.—See Ide, in *La Cellule,* iv, 1888, p. 409, and v, 1889, p. 321; also Kolossow, *Arch. mik. Anat.*, lii, 1898, p. 1. Kolossow used an osmic-acid-tannin stain, § 374.

See also Flemming, *Anat. Hefte,* 1 Abth, vi, 1895, p. 1.

Besides maceration, impregnation may be useful; Mitrophanow (*Arch. Anat. Phys., Phys. Abth.*, 1884, p. 191) has used gold chloride.

Unna (*Monatsschr. prakt. Derm.*, xxxvii, 1903, p. 1) has described a highly complicated process with Wasserblau and orcein, see *Zeit. wiss. Mik.*, xxi, 1904, p. 68.

656. Plasma-fibrils of Epithelium.—Kromayer's process (*Arch. mik. Anat.*, xxxix, 1892, p. 141) is as follows: Sections are stained for five minutes in a mixture of equal volumes of anilin water (§ 286) and concentrated aqueous solution of methyl violet 6 B. They are well washed in water and treated with solution of iodine in iodide of potassium until they become blue-black (one to thirty seconds). They are again washed with water, dried with blotting-paper, and treated with a mixture of 1 vol. of anilin to 2 vols. of xylol until sufficiently differentiated, when they are brought into pure xylol. Very thin sections will require more xylol in proportion to the anilin, viz. 1 : 3 or 1 : 4; thicker ones may require more anilin, viz. 3 : 5 or 3 : 3. Gentian or Krystall-violett will do instead of methyl violet, but not quite so well. See also Ehrmann and Jadassohn, *Arch. Dermatol. u. Syphilis*, 1892, 1, p. 303; *Zeit. wiss. Mik.*, ix, 1893, p. 356; Herxheimer, *Arch. mik. Anat.*, liii, 1899, p. 510; and Rosenstadt, *ibid.*, lxxv, 1910, p. 659 (takes the differentiating mixture much weaker in anilin).

Unna (*Monatsschr. prakt. Derm.*, xix, 1894, p. 1 and p. 277, *et seq.*; *Zeit. wiss. Mik.*, xii, 1, 1895, pp. 61, 63) has given a whole series of methods, from which the following are some extracts.

(1) Wasserblau-Orcein.—Stain sections for ten minutes in a neutral aqueous 1 per cent. solution of Wasserblau, rinse and stain for five or ten minutes in a neutral alcoholic 1 per cent. solution of Grübler's orcein. Dehydrate, clear, and mount in balsam. This may be varied as follows:

(*a*) Ten minutes in the Wasserblau and thirty minutes or more in the orcein.

(*b*) Take for the second stain an *acid* solution of orcein.

(*c*) Stain for only one minute in the Wasserblau, but for thirty or more in the neutral orcein.

(2) Stain for half an hour or more in a strong solution of hæmalum, rinse, stain for half a minute in a saturated aqueous solution of picric acid, and dehydrate for thirty seconds in alcohol containing 0·5 per cent. of picric acid.

(3) Hæmalum for two hours, neutral orcein as above for ten to twenty minutes.

More recently UNNA advocates the process mentioned last §.

See also RANVIER, *Arch. Anat. Mikr.*, iii, 1899, p. 1.

657. Keratohyalin.—The keratohyalin granules of the cells of the *stratum granulosum* are soluble in mineral acids, and can be digested in pepsin. They can be stained with picro-carmine, alum hæmatoxylin, van Gieson's mixture, or Unna's Wasserblau-orcein, last §. FICK (*Centralb. allg. Path.*, xiii, 1902, p. 987; *Zeit. wiss. Mik.*, xx, 1903, p. 222) stains sections of alcohol material for three to four minutes in concentrated aqueous solution of *Kresylechtviolett*, differentiates in alcohol, clears in xylol, and mounts in balsam.

See also UNNA, *Monatsschr. prakt. Derm.*, xx, 1895, p. 69; the article "Haut" in the *Encycl. mik. Technik.*; and UNNA and GOLODETZ, *Monatsschr. prakt. Derm.*, xlix, 1909, p. 95; LAFFONT, *Bibl. Anat.*, 1909, p. 209.

For *Trichohyalin*, see GAVAZZENI, *Monatsschr. prakt. Derm.*, xlvii, 1908, p. 229.

658. Eleidin.—To demonstrate the *stratum granulosum* and the eleidin granules RANVIER (*Arch. Anat. Micr.*, iii, 1899, p. 1) hardens with alcohol, stains with picro-carmine, and treats with lime-water. The cells swell and show up the granules, which do not change. See *loc. cit.*, other methods for the study of skin.

BUZZI (see *Encycl. mik. Technik.*, article "Haut") stains sections for a few minutes in a watchglassful of water with 2 to 3 drops of 1 per cent. Congo red. Similarly WEIDENREICH, *Arch. mik. Anat.*, lvii, 1901, p. 583. Other authors recommend nigrosin, or Wasserblau, or orcein.

See also JOSEPH, "Dermatohist. Technik," Berlin, 1905, and DREUW, *Med. Klinik*, Berlin, 1907, Nos. 27 and 28.

For *Cholesterin* see GOLODETZ and UNNA, *Monatsschr. prakt. Derm.*, xlvii, 1908, p. 1.

659. Horn, Hair, and Nails.—The elements of hairs and nails may be isolated by prolonged maceration in 40 per cent. potash solution, or by heating with concentrated sulphuric acid. See also VON NATHUSIUS, *Zool. Anz.*, xv, 1892, p. 395.

Horny tissues stain well in safranin or gentian violet (REINKE, *Arch. f. mik. Anat.*, xxx, 1887, p. 183; ERNST, *ibid.*, xlvii, 1896, p. 669; RABL, *ibid.*, xlviii, 1896, p. 489).

UNNA (*op. cit.* last §, p. 598) stains the tyrosin-bearing keratin in sections of skin for a few seconds or minutes in a mixture of 5 parts of Millon's reagent, 5 of water, and 1 of glycerin, treats shortly with nitric acid of 25 per cent., and mounts in balsam.

660. **Skin-nerves and Nerve-endings.**—Impregnate with gold chloride. See Chap. XVII, especially § 365.

661. **Tactile Corpuscles.**—See §§ 364–366.—Gold methods are indicated. See also RANVIER, *Traité*, p. 919; LANGERHANS, *Arch. mik. Anat.*, 1873, p. 730; KULTSCHIZKY, *ibid.*, 1884, p. 358; and SMIRNOW, *Intern. Monatsschr. f. Anat.*, etc., x, 1893, p. 241, who recommends, besides the gold method of Löwit, the rapid bichromate of silver method of Golgi.

662. **Corpuscles of Herbst and Corpuscles of Grandry.**—DOGIEL (*Arch. Anat. u. Entwickel.*, 1891, p. 182) has used the methylen blue method. Four per cent. solution of methylen blue, warmed to 40° C., is injected into blood-vessels of the heads of ducks or geese; pieces of skin are removed from the beaks, sectioned in pith, and the sections brought on to slides and moistened with aqueous or vitreous humour from the animal and left for ten to thirty minutes exposed to the air, then brought into picrate of ammonia, and treated as described § 343. GEBERG (*Intern. Monatsschr. Anat.*, x, 1893, p. 205) made use of a method of ARNSTEIN, according to which pieces of skin are put for twenty-four hours into lime-water, the horny layer removed, the pieces treated for five minutes with 0·25 per cent. gold chloride, reduced in water, and the precipitate that forms on them removed by putting into 0·25 per cent. cyanide of potassium and brushing.

NOWIK (*Anat. Anz.*, xxxvi, 1910, p. 217) takes UNNA'S Orceïn-wasserblau mixture (Wasserblau O.D., 1 part, orceïn 1, acetic acid 5, glycerin 20, alcohol 50, water 100) and adds to it 1 part more of orceïn. To 10 c.c. of this he adds at the moment of using 10 c.c. of 1 per cent. solution of eosin in alcohol of 80 per cent. and 3 c.c. of 1 per cent. solution of hydroquinon. Stain for five to ten minutes, rinse, stain for ten minutes in 1 per cent. aqueous solution of safranin, wash, treat for thirty minutes with 0·5 per cent. solution of bichromate of potash, dehydrate and mount.

Similarly Dogiel, *Folia Neurobiol.* iv, 1910, p. 218 (also employing Bielschowsky's neurofibril method).

663. Corpuscles of Meissner and of Krause (Cornea and Conjunctiva).—Dogiel (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 602, and xliv, 1894, p. 15) employs the methylen blue method; for details see *previous editions*.

See also Longworth's methods, *Arch. mik. Anat.*, 1875, p. 655.

664. Similar Objects.—Papillæ Foliatæ of the Rabbit, Hermann, see *Zeit. wiss. Mik.*, v, 1888, p. 524; Arnstein, *ibid.*, xiii, 1897, p. 240. **Olfactive Organs of Vertebrates,** Dogiel, *Arch. mik. Anat.*, 1887, p. 74. **Organs of a "Sixth Sense" in Amphibia,** Mitrophanow, *Zeit. wiss. Mik.*, v, 1888, p. 513 (details as to staining with "Wasserblau," for which see also *Biol. Centralb.*, vii, 1887, p. 175). **Nerve-endings in Tongue of Frog,** Fajerstain, *Arch. de Zool. expér. et gén.*, vii, 1889, p. 705. **Tongue of Rabbit,** von Lenhossek, *Zeit. wiss. Mik.*, xi, 1894, p. 377 (Ramón y Cajal's double Golgi-method).

665. Cornea.—There are three chief methods—the methylen blue, the silver, and the gold method.

For the *methylen blue method* see particularly § 345.

Negative images of the corneal cells are easily obtained by the dry *silver method* (Klein). The conjunctival epithelium should be removed by brushing from a living cornea, and the corneal surface well rubbed with a piece of lunar caustic. After half an hour the cornea may be detached and examined in distilled water.

In order to obtain *positive* images of the fixed cells the simplest plan (Ranvier) is to macerate a cornea that has been prepared as above for two or three days in distilled water. There takes place a secondary impregnation.

The same result may be obtained by cauterising the cornea of a living animal as above, but allowing it to remain on the living animal for two or three days before dissecting it out, or by treating a negatively impregnated cornea with weak salt solution or weak solution of hydrochloric acid (HIS).

But the best positive images are those furnished by *gold chloride*. RANVIER prefers his lemon-juice method. It is important that the cornea should *not remain too long in the gold solution*, or the nerves alone will be well impregnated.

ZAWARSIN (*Arch. mik. Anat.*, lxxiv, 1909, p. 116) removes the membrane of Descemet for study in the following manner. A cornea, fixed in sublimate, is dissected out and put for some hours into a mixture of alcohol and ether. Then collodion of 4 per cent. is poured on to the inner surface, and after some time a layer of collodion with the membrane attached can be peeled off, and the collodion removed from the tissue by a mixture of alcohol and ether.

See also ROLLETT, in Stricker's *Handb.*, pp. 1102, 1115, or *previous editions* ; TARTUFERI, *Anat. Anz.*, v, 1890, p. 524, or *previous editions* ; CIACCIO, *Arch. ital. Biol.*, iii, p. 75 ; and RENAUT, *C. R. Acad. Sc.*, 1880, p. 137.

666. Crystalline.—GEBHARDT (*Zeit. wiss. Mik.*, xiii, 1896, p. 306) hardens the lens for one or two days in 4 to 10 per cent. formalin; it is then easily dissociated with needles into its fibres.

RABL (*Zeit. wiss. Zool.*, lxv, 1898, p. 272) fixes the enucleated eye for half an hour in his platinum chloride or picro-sublimate, §§ 75 and 70, divides it at the equator, and puts the anterior half back for twenty-four hours into the fixative.

For **Maceration** you may use sulphuric acid, § 541.

See also ROBINSKI, *Zur Kenntniss d. Augenlinse*, Berlin, 1883.

CHAPTER XXVIII.

MUSCLE AND TENDON (NERVE-ENDINGS).

Striated Muscle.

667. Muscle-cells.—For these and allied subjects see, *inter alia*, Behrens, Kossel, und Schiefferdecker, *Das Mikroskop*, etc., vol. ii, pp. 154—161; and Schäfer, *Proc. Roy. Soc.*, xlix, 1891, p. 280.

Iron hæmatoxylin gives very fine images of striped muscle, and so does Mallory's phospho-tungstic.

For dissociation methods see §§ 527, 536, 537, 544.

To isolate the sarcolemma Solger (*Zeit. wiss. Mik*, vi, 1889, p. 189) teases fresh muscle in saturated solution of ammonium carbonate.

668. Nerve-endings—the Methylen Blue Method.—For Biedermann's procedure for the muscles of *Astacus* see § 342 (see also *Zeit. wiss. Mik.*, vi, 1889, p. 65). After impregnating as there directed the carapace should be opened, and the muscles exposed to the air in a roomy moist chamber for from two to six hours.

For *Hydrophilus piceus*, Biedermann proceeded by injecting 0·5 c.c. of methylen blue solution between the ultimate and penultimate abdominal rings, in the ventral furrow, and keeping the animals alive in water for three to four hours, then opened the thorax by two lateral incisions, and removed the muscles of the first pair of legs and exposed them to the air for three or four hours in a moist chamber, and finally examined in salt solution.

Gerlach (*Sitzb. Akad. Wiss. München*, 1889, ii, p. 125) injected frogs, either through the abdominal vein or through the aorta, with 4 to 5 c.c. of a 1 : 400 solution in 1 per cent.

salt solution, and examined pieces of muscle in serum of the animal, afterwards fixing with picrate of ammonia and mounting in glycerin jelly.

The procedure of Dogiel has been given, § 342.

669. Nerve-endings—the Gold Method.—Fischer (*Arch. mik. Anat.*, 1876, p. 365) used the method of Löwit.

Biedermann (last section) recommends for *Astacus* a similar procedure, the preliminary treatment with formic acid being omitted, and the muscles being put for a couple of days into glycerin after reduction in the acid.

Ranvier (*Traité*, p. 813) finds that for the study of the motor terminations of Vertebrates the best method is his lemon-juice process (§ 366).

See also the methods of Apáthy, §§ 368, 371.

670. Nerve-endings—the Silver Method.—Ranvier employs it as follows (*ibid.*, p. 810): Portions of muscle (gastrocnemius of frog) having been very carefully teased out in fresh serum, are treated for ten or twenty seconds with nitrate of silver solution of 2 to 3 per 1000, and exposed to bright light (direct sunlight is best) in distilled water. As soon as they have become black or brown they are brought into 1 per cent. acetic acid, where they remain until they have swelled up to their normal dimensions. They are then examined in a mixture of equal parts of glycerin and water.

This process gives *negative* images, the muscular substance being stained brown, and the nervous arborescence unstained. The gold process gives *positive* images, the nervous structures being stained dark violet.

671. Nerve-endings—the Bichromate of Silver Method.—The *rapid* method of Golgi has been used by Ramón y Cajal for the terminations of nerves and tracheæ in the muscles of insects. See *Zeit. wiss. Mik.*, vii, 1890, p. 332, or *fourth edition.* A modification is used by Wunderer, *Arch. mik. Anat.*, lxxi, 1908, p. 523.

672. Muscle-spindles.—See Cilimbaris, *Arch. mik. Anat.*, lxxv, 1910, p. 692. Principally *intra vitam* methylen blue, by injection through the internal carotid. For elastic fibres, Weigert's resorcin-fuchsin, followed by 1 per cent. orcein acidified with HCl.

Electric Organs.

673. Electric Organs.—RANVIER (*Traité*, Chap. xviii), finds that osmic acid is the only reagent that will fix properly the terminal arborisations on the lamellæ. He injects a little 2 per cent. solution under the surface of the organ, removes a small portion of it after a few minutes, and puts it into a quantity of the same solution for twenty-four hours. The electric plates may then be teased out and examined in water, and will show the stag's horn ramifications; and the dissepiments between the columns will show the bouquets of Wagner. The terminal arborescence may be impregnated with silver. A portion of the surface of the organ is rubbed with lunar caustic until it appears opaque, then removed and the plates teased out in water. This gives negative images.

Or, electric plates, isolated by teasing after twenty-four hours in osmic acid as above, and kept for some days in one-third alcohol, are washed and placed on a slide with their ventral surface uppermost. They are then treated with a few drops of 0·5 per cent. solution of chloride of gold and potassium, and those which become violet are washed and mounted in glycerin. This gives positive images.

These may also be obtained by putting material fixed by osmic acid into 2 per cent. solution of bichromate of ammonia for a few weeks, then teasing, staining with alum hæmatoxylin, and mounting in damar.

Torpedo.—BALLOWITZ (*Arch. mik. Anat.*, xlii, 1893, p. 460) gets the best results by the rapid Golgi impregnation.

An electric column, with about $\frac{1}{2}$ to 1 cm. of tissue round it, is dissected out, and put for three to four days into the osmium bichromate mixture; then for one to three days into $\frac{3}{4}$ per cent. silver, cut without imbedding and mounted in xylol balsam. Impregnates all the important elements. See further, on the whole subject, BALLOWITZ, *Encycl. mik. Techn.*, 1910, p. 298.

CAVALIÉ (*Bibl. Anat.*, xiii, 1904, p. 214) takes material fixed with osmic acid of 2 per cent. and impregnates it with gold by the method of NABIAS, and mounts in glycerin.

Raja.—IWANZOFF (*Bull. Soc. Nat. Moscou*, ix, 1895, p. 74) fixes the organ in the tail of *Raja* with liquid of Flemming, stains with hæmacalcium and eosin, and makes paraffin sections.

BALLOWITZ (*Anat. Hefte*, 1 Abth., vii, 1897, p. 285) finds the method of Golgi excellent for this organ. He also makes sections after fixing in saturated solution of sublimate (in sea-water), or in liquid of Flemming, and examines them in water. Methylen-blue may be used, *intra vitam*. Gold is little good.

Gymnotus.—BALLOWITZ (*Encycl. mik. Technik.*, p. 303) fixes with *Flemming*, and makes sections. He also commends impregnation with gold chloride, but not the Golgi method.

Malapterurus.—BALLOWITZ (*ibid.*, p. 202) fixes with picro-sublimate, with Flemming, or with various mixtures of bichromate, sublimate, and formol, and uses gold chloride and Golgi impregnations. He macerates in liquid of Müller or saturated aqueous solution of picric acid.

Tendon.

674. **Tendons.**—RETTERER (*C. R. Soc. Biol.*, x, 1898, p. 580) fixes in equal parts of saturated solutions of sublimate and picric acid, puts for 1 to 3 days into saturated picric acid with 2 to 3 per cent. of sodium chloride, to remove the mucin, and imbeds in paraffin.

674a. **Union of Muscle and Tendon.**—For this see RETTERER and LELIÈVRE, *C. R. Soc. Biol.*, 1911, No. 12 (orceïn for 24 hours, followed by iron hæmatoxylin); and SCHULTZE (*Verh. phys. med. Ges. Würzburg*, 1911, p. 33) (treats for a day or two with a mixture of equal parts of 2 per cent. bichromate of potash and alcohol, in the dark, then for 2 days with 0·5 per cent. solution of hæmatoxylin in alcohol of 70 per cent., then with Van Gieson's picro-saürefuchsin).

675. **Corpuscles of Golgi** (RANVIER, *Traité*, p. 929).—Take the tendon of the anterior and superior insertion of the gemini muscles of the rabbit. Treat it by the formic acid

and gold method (§ 365), and after reduction scrape with a scalpel, in order to remove the muscle-fibres that mask the musculo-tendinous organs.

Marchi's methods for the tendons of the motores bulbi oculi (*Archivio per le Scienze Mediche*, vol. v, No. 15).—The enucleated eyes, together with their muscles, were put for not less than three days into 2 per cent. bichromate of potash. The muscles and tendons were then carefully dissected out, stained with gold chloride and osmic acid (Golgi's method), and by the method of Manfredi, § 368. Mount in glycerin. The methods only succeed completely during fine, sunny weather.

Ruffini (*Atti R. Acc. Lincei Roma Rend.* [5], i, 1892, p. 442) recommends the method of Fischer.

Ciaccio (*Mem. R. Acc. Sci. Bologna* [4], t. x, 1890, p. 301) puts tendons of Amphibia into 0·1 per cent. hydrochloric acid or 0·2 per cent. acetic acid until transparent; then for five minutes into a mixture of 0·1 per cent. gold chloride and 0·1 per cent. potassium chloride; then back into the acetic acid, for a day in the dark, and for two or three hours more in the sunlight. When they have become somewhat violet they are put for a day into 0·1 per cent. osmic acid, and finally mounted in glycerin acidulated with 0·5 per cent. of acetic or formic acid.

Dogiel (*Arch. mik. Anat.*, lxvii, 1906, p 638) stretches tendons of eye-muscles on cardboard with hedgehog spines, puts for 4 or 5 days into nitrate of silver of 1 to 2 per cent., reduces for a day in pyrogallic acid with formol, and imbeds in celloidin.

Smooth Muscle.

676. Tests for Smooth Muscle.—Picro-säurefuchsin, § 299, stains muscle yellow, connective tissue red.

Picro-nigrosin, § 325, stains muscle yellowish, connective tissue blue.

Unna (*Encycl. mik. Technik.*, article "Kollagen") stains for twenty-four hours in orceïn 1 part, Wasserblau 0·25, alcohol 60, glycerin 10, water 30, which gives muscle in a mixed tone, collagen blue, elastin reddish. See also a complicated process with methylen blue in *Monatssch. prakt.*

Dermatol., xix, 1894, p. 533, and another with orcein, hæmatein, säurefuchsin and picric acid.

Retterer (*C. R. Soc. Biol.*, 1887, p. 645) fixes in 10 vols. of alcohol with one of formic acid, washes well and stains in alum carmine. Muscle red, connective tissue unstained.

677. General Structure.—Werner (*Hist. d. glatten Musculatur*, Dorpat, 1894, p. 22) fixes stretched intestine or bladder in Flemming, washes well and stains in Heidenhain's chrome hæmatoxylin, § 265. For demonstrating intercellular spaces, fresh intestine is put for 24 hours into oil, at 37° C., then for 12 hours into Flemming, and for 4 to 6 into chromo-acetic acid.

678. Isolation of Fibres.—Gage's methods, see §§ 527, 544, and 536.

Mobius, muscle of *Cardium*, see § 535.

Ballowitz, muscle of Cephalopoda, see *Arch. mik. Anat.*, xxxix, 1892, p. 291.

Schultz (*Arch. Anat. Phys., Phys. Abth.*, 1895–6, p. 521) puts muscle of Vertebrates for twenty-four hours into 10 per cent. nitric acid, rinses with water, and brings pieces for six to eight days (in the dark at first) into a mixture of equal parts of $\frac{1}{20}$ per cent. osmic acid and $\frac{1}{5}$ per cent. acetic acid, teases and mounts in glycerin.

For smooth muscle of Vermes, see Apáthy, *Zeit. f. wiss. Mik.*, x, 1893, pp. 36, 319, and § 538, *ante*.

679. Iris.—Dogiel (*Arch. mik. Anat.*, 1886, p. 403) puts the anterior half of an enucleated eye for some days into a mixture of two parts one-third alcohol and one part 0·5 per cent. acetic acid. The iris can then be isolated, and split from the edge into an interior and posterior plate, and these stained according to the usual methods.

See also Koganei, *Arch. mik. Anat.*, 1885, p. 1; Canfield, *ibid.*, 1886, p. 121; and Dostoiewsky, *ibid.*, p. 91.

680. Bladder of Frog, Innervation of (Wolff, *Arch. f. mik. Anat.*, 1881, p. 362).—A frog is killed and a solution of gold chloride of 1 : 20,000 injected into the bladder through the anus. (If the injection flows out on removal of the syringe, tie the frog's thighs together.) Now open the frog, ligature the intestine above the bladder, and cut away the abdomen so as to have in one piece bladder, rectum and hind legs. Put this into gold solution of 1 : 2000 for four hours; the bladder is then excised, slit open, and pinned (with hedgehog spines) on to a cork (outside downwards). Place it under running water until all the epithelium is washed away. Use a camel's-hair brush if necessary. Put for twenty-four hours into gold solution of 1 : 6000. Wash in pure water, and put away in the dark "for some time" in acidulated water, and finally reduce in fresh water in daylight.

Ranvier (*Traité*, p. 854) recommends his two gold processes, the liquids being injected as above.

Grünstein (*Arch. mik. Anat.*, 1899, p. 1) injects 1 per cent. methylen blue in normal salt solution through the *vena abdominalis*, and after twenty to thirty minutes excises the bladder and exposes to the air. Fix the stain with picrate of ammonia and mount in glycerin with the same (§ 343).

CHAPTER XXIX.

CONNECTIVE TISSUES.

Connective Tissue.

681. General Stains for Connective Tissue.—Connective tissue, elastic tissue, and smooth muscle are all *normally* acidophilous. Collagen, the distinctive element of connective tissue, absolutely requires "acid" dyes for the production of a permanent stain, whilst elastic tissue and muscle will also fix "basic" dyes. Collagen has a special affinity for Säurefuchsin and Wasserblau. Elastin has a strong affinity for acid orcein, whilst muscle has no special affinity for either, but stains energetically with picric acid.

Picro-säurefuchsin is much used and very convenient as a general differentiating stain, but not to be recommended for cytological detail. See Schaffer, *Zeit. wiss. Zool.*, lxxx, 1905, p. 176.

E. and T. Savini recommend Benda's picro-säurefuchsin, § 299.

Ehrlich-Biondi mixture gives connective tissue red, but smooth muscle redder still.

Unna's *Wasserblau-orcein* for distinguishing connective tissue and muscle has been given, § 676. It works after all fixatives. Stain long, and dehydrate preferably with acid alcohol.

Freeborn (*Amer. Mon. Mic. Journ.*, 1888, p. 231) recommends (for sections) *picro-nigrosin*, made by mixing 5 c c. of 1 per cent. aqueous solution of nigrosin, with 45 c.c. of aqueous solution of picric acid. Stain for three to five minutes, wash with water, and mount in balsam. Connective tissue blue, nuclei blackish, the rest yellowish.

Ramón y Cajal's *picro-indigo-carmine* gives connective-tissue fibres dark blue, with red nuclei.

S. Mayer (*Sitzb. k. Akad. Wiss.*, lxxxv, 1882, p. 69) recommends for staining *fresh* tissue Violet B, § 330. Elastic fibres and smooth muscle also stain, but of different tints.

DUBREUIL (*C. R. Ass. Anat.*, vi Sess., 1904, p. 62) uses a mixture of 23 vols. 1 per cent. picric acid and 2 vols. 1 per cent. methyl blue—with a foregoing stain with carmalum or safranin.

For RANVIER's method of artificial œdemata for the study of areolar tissue, see his *Traité*, p. 329.

682. UNNA's **Orcein Method.**—(*Encycl. mik. Techn.*, 1910, p. 250). Sections are stained for ten minutes in Grübler's polychrome methylen blue. They are then washed with water, mopped up, and brought for fifteen minutes into a *neutral* 1 per cent. solution of orcein in absolute alcohol, rinsed in pure alcohol, cleared in bergamot oil, and mounted. Collagenous ground-substance dark red, muscle bluish, elastic fibres *sometimes* dark red. Material may be fixed in almost any way except with nitric or picric acid, formol, or liquids of Müller and Hermann.

683. UNNA's **Methylen-blue + Säurefuchsin** (UNNA, in *Encycl. mik. Technik*, 1910, p. 247). Stain for 2–5 minutes in polychrome methylen blue solution (Grübler). Wash and stain for 10–15 minutes in "(0·5 per cent.) Säurefuchsin + (33 per cent.) tannin-mixture (Grubler)." Water, alcohol, essence, balsam. Collagen, protoplasm, and muscle red, nuclei and keratin blue. On Flemming material, elastin blue. Liquids of Hermann and Erlicki, formol and copper fixatives incompatible.

684. UNNA's **Safranin + Wasserblau** (*ibid.*). Ten minutes in 1 per cent. safranin. Wash. Ten to 15 minutes in (" 1 per cent.) Wasserblau + (33 per cent.) tannin mixture." Wash. Stains in opposite colours to the last. Formol and liquid of Hermann contra-indicated for fixing.

685. **Flemming's Orange Method** is said to give a very sharp differentiation of *developing fibrils.*

686. MALLORY (*Zeit. wiss. Mik.*, xviii, 1901, p. 175) stains sections of sublimate or Zenker material for a few minutes in Saürefuchsin of 0·1 per cent., mordants for a few minutes in 1 per cent. phosphomolybdic

acid and stains for 2 to 20 minutes in anilin blue 0·5 grms., Orange G. 2, oxalic acid 2, and water 100. His phosphotungstic hæmatoxylin stains connective tissue sharply, but does not differentiate it sufficiently from elastic tissue and muscle.

687. For the complicated procedure of HORNOWSKI see *ibid.*, xxvi, 1909, p. 138.

688. For DELAMARE'S mixture of orcein, hæmatoxylin, Säurefuchsin and picric acid see *Verh. Anat. Ges.*, xix, 1905, p. 227.

689. MASSON (*C. R. Soc. Biol.*, lxx, 1911, p. 573), stains first in hæmalum, then in eosin, and then for a few minutes in 1 per cent. solution of saffron in tap water (made by boiling). Connective tissue, bone, and cartilage, yellow.

690. **Benecke's** stain for fibrils (*Verh. Anat. Ges.*, vii, 1893, p. 165) is essentially that of KROMAYER, § 656.

691. **Bielschowsky's** SILVER METHOD (*post, under 'Neurofibrils'*) has been used for connective-tissue fibrils. SNESSAREW (*Anat. Anz.*, xxxvi, 1910, p. 401) employs it as follows: Tissue is hardened in neutral formol and sectioned with a freezing microtome. The sections are put for at least 4 days into *iron alum* of 2·5 to 10 per cent., changed daily. They are then silvered for 36 to 48 hours in nitrate of silver of 10 per cent., then treated with the oxide bath and reduced in formol of 20 per cent. Collagen fibres grey, but fine connective networks black, nerve fibres unstained or only weakly stained.

See also MARESCH, *Zeit. wiss. Mik.*, xxiii, 1906, p. 356; STUDNICKA, *ibid.*, p. 416; ZIMMERMANN, *ibid.*, xxv, 1908, p. 10; LEVI, *Monit. zool. Ital.*, 1908, p. 290; HEINRICH, *Arch. Mik. Anat.*, lxxiv, 1909, p. 786 (dentine): INSABATO, *Arch. Ital. Anat. Emb.*, viii, 1909, p. 375 (silvers Flemming material); ATHANASIU and DRAGOIU, *C. R. Acad. Sci.*, cli, 1910, p. 551 (Ramón y Cajal's silver process, with alcohol fixation).

Elastic Tissue.

692. **Elastic Tissue, Generalities.**—Elastic fibres have a great affinity for osmium, staining with much more rapidity than most other tissue elements. They are not changed by

caustic soda or potash. They are *normally* acidophilous, but are easily rendered *artificially* basophilous by means of chromic acid or other mordants, and then stain with great energy with basic dyes. Hence a group of stains of which those of Lustgarten and Martinotti are types. They have a natural affinity for orcein, whence stains of the Taenzer-Unna type.

For a review of the older methods of BALZER, UNNA, LUSTGARTEN, and HERXHEIMER, see the paper by G. MARTINOTTI, in *Zeit. wiss. Mik.*, iv, 1887, p. 31; also *Encycl. mik. Technik.*, art. "Elastin."

693. Victoria Blue (LUSTGARTEN). See § 289.

694. Safranin (G. MARTINOTTI, *loc. cit.*, § 692).—Fix in a chromic liquid, wash, stain for forty-eight hours in strong (5 per cent. Pfitzner's) solution of safranin, wash, dehydrate, clear, and mount in balsam. Elastic fibres black.

The staining will be performed quicker if it be done at the temperature of an incubating stove (GRIESBACH, *ibid.*, iv, 1887, p. 442). See also FERRIA (*ibid.*, v, 1888, p. 342).

See also MIBELLI, *Mon. Zool. Italiano*, 1, p. 17, or *Zeit. wiss. Mik.*, vii, 1890, p. 225 (the report in *Journ. Roy. Mic. Soc.*, 1890, p. 803, is vitiated by a misprint). Other basic dyes have been recommended.

695. Kresofuchsin (RÖTHIG, see § 289).

696. Orcein.—This method is due to TAENZER, and as modified by UNNA is known as the TAENZER-UNNA method, see *third edition*, or *Monatssch. prakt. Dermatol.*, xii, 1891, p. 394.

UNNA'S **Modified Orcein Method** (*Monatssch. prakt. Dermatol.*, xix, 1894, p. 397; *Zeit. wiss. Mik.*, xii, 1895, p. 240).—Grübler's orcein 1 part, hydrochloric acid 1 part, absolute alcohol 100 parts. Stain sections for thirty to sixty minutes, or for ten to fifteen at 30° C., rinse in alcohol, clear, and mount. Elastin dark brown, collagen light brown.

See also *Merk. Silz. Akad. Wiss. Wien.*, cviii, 1899, p. 335; PRANTER, *ibid.*, xix, 1903, p. 361 (he takes 2 per cent. of nitric acid instead of the hydrochloric, and stains six to twenty-four hours); WOLFF, *ibid.*, p. 488; the article "Elastin" in *Encycl. mik. Technik.*; and E. and T. SAVINI, *Zeit. wiss. Mik.*, xxvi, 1909, p. 34.

697. Weigert's Resorcin-Fuchsin Method (*Centralb. allg. Path.*, ix, 1898, p. 290).—1 per cent. of basic fuchsin and 2 per cent. of resorcin (or of carbolic acid) are dissolved in water. 200 c.c. of the solution are raised to boiling-point in a capsule, and 25 c.c. of *Liquor ferri sesquichlorati* P. G. are added, and the whole is boiled, with stirring, for two to five minutes more. A precipitate is formed. After cooling the liquid is filtered, and the precipitate which remains on the filter is brought back into the capsule, and there boiled with 200 c.c. of 94 per cent. alcohol. Allow to cool, filter, make up the filtrate to 200 c.c. with alcohol, and add 4 c.c. of hydrochloric acid.

Wolfrum (*Zeit. wiss. Mik.*, xxv, 1908, p. 219) adds 10 to 15 per cent. of acetone to the mixture.

Stain sections (of material fixed in any way) for twenty minutes to an hour, wash with alcohol, clear with xylol (not with an essence). Elastic fibres dark blue on a light ground.

Minervini (*Zeit. wiss. Mik.*, xviii, 1901, p. 161) gives a variant with safranin instead of fuchsin.

See also Pranter, *ibid.*, xix, 1903, p. 361; B. Fischer, *Virchow's Arch.*, clxx, 1902, p. 285, or *Zeit. wiss. Mik.*, xx, 1903, p. 40 (chemistry of the dyes obtained by these processes, which he calls "Fuchselin," "Safranelin," etc.); Hart, *Centralb. allg. Path.*, xix, 1908, p. 1; and Cilimbaris, *Arch. mik. Anat.*, lxxv, 1910, p. 708.

698. Hæmatoxylin Methods.—Harris (*Zeit. wiss. Mik.*, xviii, 1902, p. 290) makes an "Elasthæmatein" as follows: Hæmatoxylin 0·2 grms., aluminium chloride 0·1 grms., alcohol of 50 per cent. 100 c.c., boil and add mercuric oxide 0·6 grms., filter and add 1 drop of HCl. Keep for some weeks. Stain for five or ten minutes, put into alcohol with 1 per cent. of nitric acid for one minute, then pure alcohol.

See also De Witt, *Anat. Rec.*, i, 1897, p. 74; Duerck, *Arch. Path. Anat.*, clxxxix, 1907, p. 62; Verhoeff, *Journ. Amer. Med. Assoc.*, 1908, No. 11.

Mallory's phosphotungstic hæmatoxylin is good, but not specific.

699. Other Methods for Elastic Tissue:

For the elastic tissue of the skin see Passarge and Krösing, *Derm. Stud.*, xviii, 1894.

See also for staining and dissociation Agababow, *Arch. mik. Anat.*, l, 1897, p. 566 *et seq.*

For C. Martinotti's silver impregnation see *Zeit. wiss. Mik.*, v, 1888, p. 521, or *Arch. Ital. Biol.*, xi, 1889, p. 257.

Schumacher (*Arch. mik. Anat.*, lv, 1899, p. 151) has had good results (for the spleen) with picro-nigrosin, § 681.

See also § 733.

Plasma Cells.

700. Plasma Cells and "Mastzellen"; Generalities.—Plasma cells, of which "Mastzellen" are a sub-species, are cells found in or along with connective tissue, and distinguished by their hypertrophied and very granular cytoplasm and poorly staining nucleus. The granules are highly basophilous, much more so than the nuclei; they stain with special energy with basic anilins, and mostly metachromatically. They do not, however, stain with pure methyl green. The nuclei either do not stain at all or not in the normal way, except with pure methyl green.

According to UNNA in *Encycl. mik. Techn.*, 1910, ii, p. 411, material should be fixed in *chemically pure* absolute alcohol and sectioned in celloidin. Care should be taken to avoid contamination of the liquids by tannin; corks, and supports for imbedding, should be soaked for some hours before use in 2 per cent. carbonate of soda.

701. Mastzellen (NORDMANN, *Beitr. z. Kenntniss d. Mastzellen, Inaugural diss.*, Helmstedt, 1884).—A concentrated solution of vesuvin containing 4 to 5 per cent. of hydrochloric acid. Stain for a few minutes, and dehydrate with absolute alcohol.

702. Plasma Cells, UNNA's Later Methods (UNNA, in *Encycl. mik. Techn.*, 1910, ii, p. 411).

A.—For Large Plasma Cells.

(1) Ten minutes in Grübler's polychrome methylen blue solution, wash and drain. Fifteen minutes in 1 per cent. orcein solution (Grübler), *without acid*; absolute alcohol, so long as methylen blue comes away abundantly; bergamot oil, balsam.

(2) Methylen blue as above, 2 minutes. Wash well. Then two minutes in glycerin-ether mixture* (Grübler) diluted with 4 volumes of water. Wash thoroughly (2 to 5 minutes); absolute alcohol, bergamot oil, balsam.

(3) Modification of a method of PAPPENHEIM (*Virchow's*

---

* Glycerin ether $C_6H_{10}O_3$, is a glycerin anhydride. It is a differentiating agent for basic dyes. The glycerin-ether mixture in question contains alcohol and glycerin, and can be obtained from Grübler.

Arch., clxiv, 1901, p. 111). Ten minutes in the warm, 20° to 40° C., in Grübler's carbol-pyronin-methyl-green mixture. *Cool rapidly*, by plunging the recipient containing the tissues into cold water. Remove the tissues with a platinum wire and rinse. Absolute alcohol, bergamot oil, balsam.

Stropeni (*Zeit. wiss. Mik.*, xxix, 1913, p. 302) takes acridin red instead of the pyronin. This will work after various fixatives.

B.—For small Plasma Cells.

(4) As No. 2, *supra*, but only half a minute in the glycerin-ether.

(5) After removal of the celloidin from the sections with alcohol and ether, five minutes in polychrome methylen blue, wash, dry with blotting-paper, dehydrate (about a minute) in a mixture of 2 parts alcohol to 3 of xylol, then one minute in xylol; then 5 to 10 minutes in alum-anilin (prepared by allowing anilin oil to stand over a layer of powdered alum a couple of fingers deep); xylol, balsam.

(6) As No. 3, *supra*, after a foregoing stain of two minutes in polychrome methylen blue.

See also Ehrlich in *Virchow's Arch.*, clxxv, 1904, p. 198.

703. Ehrlich's **Original Method for Mastzellen** (*Arch. mik. Anat.*, xii, 1876, p. 263).—Stain, for at least twelve hours in—

Absolute alcohol	50 c.c.
Water	100 c.c.
Acid. acet. glacial	12½ c.c.

—to which has been added enough dahlia to give an almost saturated solution. Wash out with alcohol, and mount in resinified turpentine.

See also Schiefferdecker and Kossel's *Gewebelehre*, p. 329.

704. Mastzellen, Unna's **Latest Methods** (*Encycl. mik. Techn.*, 1910, ii, p. 72).—(1) Stain three hours to overnight in polychrome methylen blue with a knife-pointful of alum to a watch-glass of the stain, rinse; alcohol, oil, balsam. (2) Stain in polychrome methylen blue quarter of an hour, rinse, then ten minutes in glycerin-ether, § 702, wash thoroughly, alcohol, oil, balsam.

These methods give a specific metachromatic stain of Mastzellen on a light ground. See also *loc. cit.*, two other methods demonstrating plasma cells at the same time.

705. Other Methods for Plasma Cells and Mastzellen.—See, *inter alios* (in *previous editions*) PAPPENHEIM, *Virchow's Arch.*, clxvi, 1901, p. 427; BERGONZINI, *Anat. Anz.*, 1891, p. 596; SCHRIDDE, *Anat. Hefte*, xxviii, 1905, p. 698; MAXIMOW, *Arch. mik. Anat.*, lxvii, 1906, p. 686; SCHAFFER, *Centrabl. Phys.*, xxi, 1907, p. 261 (fixation in absolute alcohol or 2 parts alcohol to 1 of formol, staining for half an hour in methylen blue, thionin or toluidin blue, in 70 per cent. alcohol with 1 per cent. of HCl); RANVIER, *C. R. Acad. Sci.*, 1890, p. 165 (his "Clasmatocytes": fix with osmium, stain with aqueous methyl violet 5B).

Fat.

706. Fat and Lecithin.—According to LOISEL (*C. R. Soc. Biol.*, lv, 1903, p. 703) lecithin is much less soluble than neutral fats in turpentine or ether, and in general much more stainable. To demonstrate lecithin, he advises as follows: After fixation, mordant the tissues with alum; leave them as little as possible in alcohol; dehydrate with acetone, ether, or benzin; stain with hæmatoxylin, gentian, methyl green, toluidin blue, Säurefuchsin or orange G, which stain lecithin strongly, whilst leaving fat unstained.

DEFLANDRE (*Journ. Anat. Phys.*, 1904, p. 80) fixes in formol of 4 per cent. and brings into acetone, in which fat is dissolved, but not lecithin, which can then be stained by osmium.

See also CIACCIO, *Arch. Zellforsch.*, v, 1910, p. 235; and FISCHLER, *Zeit. wiss. Mik.*, xxii, 1905, p. 262.

707. Mounting Fat.—*Osmicated* fat can generally be mounted in balsam without special precaution. For delicate fats, it may suffice to avoid absolute alcohol and essences as much as possible, and mount direct in *alcohol balsam* or *euparal*, or clear with cedar oil, which has little solvent action. For very delicate fats it may be necessary to avoid alcohol of more than 70 per cent., or avoid it altogether, and mount in glycerin or levulose.

708. Removal of Fat.—Fat can generally be dissolved out by alcohol, ether, or chloroform and the like. *Osmicated* fat is more resistant, but can be removed in a few hours or days by means of oil of turpentine, ether, creosote, xylol, clove oil, of chloroform. See FLEMMING in *Zeit. wiss. Mikr.*, 1889, pp. 39, 178.

709. **Stains for Fat.**—The simplest, and perhaps the best is osmic acid. This stains *certain* fatty bodies black, but not all.

According to ALTMANN, STARKE, and HANDWERCK, only free oleic acid and olein are directly blackened by osmic acid; stearin and palmitin, and stearic and palmitic acid are only browned by it, with an after-blackening which is produced by subsequent treatment with alcohol. Neither reaction occurs with the fatty bodies in the solid state, and can only be obtained when they are either in a state of fusion or solution (from the paper of HANDWERCK in *Zeit. wiss. Mik.*, xv, 1898, p. 177). See also LOISEL, *C. R., Soc. Biol.*, 1903, p. 826. MULON, *Zeit. wiss. Mik.*, xxii, 1905, p. 138; GOLODETZ, *Chem. Rev. Fett. u Harz-Industrie*, xvii, 1910, p. 70 (*Zeit. wiss. Mik.*, xxviii, 1911, p. 213).

For *quinolein blue*, see § 322.

DADDI (*Arch. Ital. Biol.*, xxvi, 1896, p. 143) stains fat in tissues by treating for 5 to 10 minutes with concentrated alcoholic solution of **Sudan III**, washing for the same time with alcohol, mopping up with blotting paper, and mounting in glycerine.

Similarly RIEDER, see *Zeit. wiss. Mikr.*, xv, 1898, p. 211.

The alcohol for making the stain should be of 70 per cent., according to most authors, though SATA (*Beitr. path. Anat.*, xxviii, 1900, p. 461; *Zeit. wiss. Mik.*, xviii, 1901, p. 67) employs 96 per cent. ROSENTHAL (*ibid.*, xix, p. 469; *Verh. path. Ges.*, September, 1899, p. 440) insists that the washing-out be done with alcohol of exactly 50 per cent.

MICHAELIS (*Virchow's Arch.*, clxiv, 1901, p. 263) recommends **Scharlach R** (syn. "Fettponceau"). Stain for fifteen to thirty minutes in a saturated solution in 70 per cent. alcohol, and mount in glycerin or levulose.

Other authors also commend this stain. HERXHEIMER (*Deutsche med. Wochenschr.*, xxvii, 1901, p. 607; *Zeit. wiss. Mik.*, xix, 1902, p. 66) makes a solution of 70 parts of absolute alcohol, 10 of water, 20 of 10 per cent. caustic soda, and Scharlach R to saturation. This makes a stronger solution, and stains in a couple of minutes. Wash out with alcohol of 70 per cent.

With either solution the staining must be done in a covered vessel or the stain will precipitate.

Similarly BELL, *Amer. Journ. Anat.*, ix, 1909, p. 401, and *Anat. Rec.*, iv, 1910, p. 199.

HERXHEIMER also (*Centralb. allg. Path.*, xiv, 1903, p. 841; *Zeit. wiss. Mik.*, xxi, 1904, p. 57) recommends a saturated solution of the dye in a mixture of equal parts of acetone and 70 per cent. alcohol.

He also (*Deutsche med. Wochenschr.*, xxvii, 1901, p. 607; *Zeit. wiss. Mik.*, xix, 1902, p. 67) has had very fine results by staining for twenty minutes in a saturated solution of **Indophenol** in 70 per cent. alcohol.

MOLLISON (*Zeit. wiss. Zool.*, lxxvii, 1904, p. 529) has had good results by staining gelatin sections for a few minutes in strong extract of **Alkanna** in 96 per cent. alcohol, and mounting in glycerin or syrup.

LORRAIN SMITH (*Journ. Path. Bact.*, xii, 1907, p. 1) finds that **Nile blue** stains fatty acids blue and neutral fats reddish.

Similarly EISENBERG (*Virchow's Arch.*, cxcix, 1910, p. 502) who recommends aqueous solution of **Nilblau BB.**

BENDA (*ibid.*, clxi, 1900, p. 194) finds that free fatty acids can be detected by Weigert's neuroglia mordant. See also BERNER, *ibid.*, clxxxvii, 1907, p. 360, and FISCHLER, *Zeit. wiss. Mik.*, xxii, 1905, p. 263.

OKAJIMA (*ibid.*, xxix, 1912, p. 67) extracts red **capsicum berries** for some days with alcohol, and evaporates down to one fifth. This stains only fatty bodies: amongst them, myelin.

See also KINGSBURY, *Anat. Rec.*, v, 1911, p. 313.

Bone.[*]

710. Bone, Non-decalcified.—RANVIER (*Traité*, p. 297) has the following:

Bones should be plunged into water, without being allowed to dry, as soon as the surrounding soft parts have been removed, and should be divided into lengths with a saw whilst wet. The medulla should then be driven out from the central canal by means of a jet of water; spongy bones should be treated as follows:

---

[*] For a detailed review of the whole subject, see the paper of SCHAFFER in *Zeit. wiss. Mik.*, x, 1893, p. 167, or the article "Knochen und Zähne" in *Encycl. mik. Technik.*

An epiphysis having been removed, together with a small portion of the diaphysis, a piece of caoutchouc tubing is fixed by a ligature on to the cut end of the diaphysis, and the free end of the piece of tubing adapted to a tap through which water flows under pressure; they are then put to macerate for several months, the liquid being changed from time to time. As soon as all the soft parts are perfectly destroyed, the bones may be left to dry.

Thin sections may then be cut with a saw and prepared by rubbing down with pumice-stone. Compact pumice-stone should be taken and cut in the direction of its fibres. The surface should be moistened with water and the section of bone rubbed down on it with the fingers. When both sides of the sections have been rubbed smooth in this way, another pumice-stone may be taken, the section placed between the two, and the rubbing continued. As soon as the section is thin enough to be almost transparent it is polished by rubbing with water (with the fingers) on a Turkey hone or lithographic stone. Spongy bone should be soaked in gum and dried before rubbing down (but see VON KOCH's copal process, and EHRENBAUM's colophonium process).

SCHAFFER (*Zeit. wiss. Mik.*, x, 1893, p. 171) grinds and polishes on stones of graduated fineness.

For the process of WEIL for bones and teeth see § 180.

RÖSE (*Anat. Anz.*, vii, 1892, pp. 512–519) follows Koch's process. He penetrates first with a mixture of cedar oil and xylol, then with pure xylol, and imbeds in solution of Damar in chloroform or xylol. The method can be combined with Golgi's impregnation.

WHITE (*Journ. Roy. Mic. Soc.*, 1891, p. 307) recommends the following: Sections of osseous or dental tissue should be cut or ground down moderately thin, and soaked in ether for twenty-four hours or more. They should then be put for two or three days into a thin solution of fuchsin in collodion, then into spirit to harden the collodion. After this they are ground down to the requisite thinness between two plates of old ground glass, with water and pumice powder, and mounted, *surface dry*, in stiff balsam or styrax, care being taken to use as little heat as possible. Lacunæ, canaliculi, and dentinal tubuli are found infiltrated by the coloured collodion.

MATSCHINSKY (*Arch. mik. Anat.*, xxxix, 1892, p. 151, and xlvi, 1895, p. 290), after grinding, impregnates with nitrate of silver.

For similar method of RUPRECHT, see *Zeit wiss. Mik.*, xiii, 1896, p. 21, wherein see also quoted (p. 23) a method of ZIMMERMANN.

CSOKOR (*Verh. anat. Ges.*, 1892, p. 270) describes a saw which will cut *fresh* bone to $120\,\mu$; and ARNDT (*Zeit. wiss. Mik.*, xviii, 1901, p. 146) a double saw which will also give very thin sections.

711. Mounting.—To show lacunæ and canaliculi injected with air, take a section, or piece of very thin flat bone, quite dry. Place on a slide a small lump of solid balsam, and apply just enough heat to melt it. Do the same with a cover glass, place the bone in the balsam, cover, and cool rapidly.

712. Sections of Bones or Teeth showing the Soft Parts.—NEALEY (*Amer. Mon. Mic. Journ.*, 1884, p. 142; *Journ. Roy. Mic. Soc.*, 1885, p. 348) says that perfectly *fresh* portions of bone or teeth may be ground with emery on a dentist's lathe, and good sections, with the soft parts *in situ*, obtained in half an hour.

HOPEWELL-SMITH (*Journ. Brit. Dent. Ass.*, xi, 1890, p. 310; *Journ. Roy. Mic. Soc.*, 1890, p. 529) says that for preparing sections of teeth showing odontoblasts *in situ* the best plan is to take embryonic tissues. A lower jaw of an embryonic kitten or pup may be taken, and hardened in solution of Müller followed by alcohol, then cut with a freezing microtome.

WEIL (*loc. cit.*, § 180) fixes pieces of fresh teeth in sublimate, stains with borax-carmine, brings them through alcohol into chloroform and chloroform-balsam, and after hardening this by heat proceeds to grind as usual (§ 177).

See also RÖSE, § 710.

For the study of the *vessels in teeth*, LEPKOWSKY (*Anat. Hefte*, viii, 1897, p. 568) injects with Berlin blue, hardens the teeth with a piece of the jaw for one or two days in 50 per cent. formol, decalcifies in 10 per cent. nitric acid (eight to fourteen days, change frequently) and makes celloidin sections.

For decalcification of teeth see also § 554 (ROUSSEAU, BÖDECKER and FLEISCHMANN). Bödecker finds Rousseau's process not applicable to *human* teeth : the acid *must* be added to the *fluid* celloidin.

713. VIVANTE (*Intern. Monasschr. Anat. u. Phys.*, ix, 1892, p. 398) impregnates portions of frontal bone of four to six months calves, which are not more than 3 to 4 mm. thick, by Golgi's rapid bicromate and silver process. After impregnation the specimens should be decalcified in von Ebner's mixture (§ 562), well washed with water, and brought into solution of carbonate of soda, and finally imbedded in paraffin. For his quinoleïn blue method see *fourth edition.*

For UNDERWOOD'S gold process for teeth, and for that of LEPKOWSKI, see *third edition,* or *Anat. Anz.*, 1892, p. 294.

LAW (*Proc. Roy. Soc. Med.*, i, 1908, p. 45) studies nerve-endings in teeth of mammals by treating paraffin sections of decalcified tissue with BETHE'S molybdenum toluidin blue (details in *Journ. Roy. Micr. Soc.*, 1908, p. 518).

714. **Bone, Decalcified** (FLEMMING, *Zeit. wiss. mik.*, 1886, p. 47).—Sections of decalcified bone are soaked in water, dehydrated with alcohol, under pressure, dried under pressure and mounted in hard balsam melted on the slide. They show the lacunar system injected with air as in non-decalcified sections (§ 711).

715. **Stains for Cartilage and Decalcified Bone.**—See hereon SCHAFFER in *Zeit. wiss. Mik.*, v, 1888, p. 1 ; and *Encycl. mik. Technik.*, art. "Knochen."

KÖLLIKER (*Zeit. wiss. Zool.*, xliv, 1886, p. 662) treats sections of decalcified bone with concentrated acetic acid until they become transparent, and then puts for one quarter to one minute into a concentrated solution of indigo-carmine, washes and mounts in glycerin or balsam. The fibres of Sharpey appear *red,* the remaining bone substance blue.

SCHAFFER (*Zeit. wiss. Mik.*, v, 1888, p. 17) employed at one time a safranin method modified from BOUMA (*Centralb. med. Wiss.*, 1883, p. 866), for which see *previous editions.* He now (*Encycl. mik. Tech.*, 1910, i, p. 762) stains sections for twenty-four hours in a bath of 20 c.c. of water with one drop of 1 per cent. solution of safranin (or thionin) and (apparently) mounts in balsam. The safranin stain will keep if the material is cartilage which has been fixed in picro-sublimate; otherwise it must be fixed with ammonium molybdate of 5 per cent. before dehydrating.

SCHMORL (*Centralb. allg. Path.*, x, 1899, p. 745) stains in a mixture of 2 c.c. concentrated solution of thionin in alcohol of 50 per cent. and 10 c.c. of water for ten minutes, rinses and puts into saturated aqueous picric acid for thirty to sixty seconds. Rinse and pass through graded alcohols into origanum oil or carbol-xylol and balsam. Matrix yellow, cells red, fat-cells violet. He also describes a more complicated method with thionin and phosphotungstic or phosphomolybdic acid.

MOLL (*Centralb. Physiol.*, xiii, 1899, p. 225) stains embryonic cartilage for six to twenty-four hours in orcein 0·5 gr., alcohol 40, water 20, hydrochloric acid 20 drops, and mounts in balsam. Matrix blue, nuclei red.

KALLIUS (*Anat. Hefte*, xxx, 1905, p. 9) stains first with borax carmine or alum-carmine, then (sections) for ten minutes in saturated solution of thionin, and washes out with alcohol of 70 per cent. Said to be specific for embryonic cartilage.

VASTARINI-CRESI (*Att. Accad. med.-chir. Napoli*, 1907, p. 4) stains sections of embryonic cartilage with borax carmine, then with muchæmatein (alcoholic solution without acid), and then with orange G. in alcohol.

BAYERL'S *method for ossifying cartilage* (*Arch. mik. Anat.*, 1885, p. 35):—Portions of ossified cartilage are decalcified as directed § 563, cut in paraffin, stained in Merkel's carmine and indigo-carmine mixture, and mounted in balsam.

MAYER (*Grundzüge*, LEE & MAYER, 1910, p. 393) prefers to all these *resorcin fuchsin*, § 697, the precipitate being freed from iron chloride by washing before dissolving in the alcohol.

Aqueous solution of *benzoazurin* has been commended as a stain for ossifying cartilage by ZSCHOKKE, see *Zeit. wiss. Mik.*, x, 1893, p. 381.

A process of BAUMGARTEN'S has been given, § 388.

MOERNER (*Skandinavisches Arch. Physiol.*, i, 1889, p. 216; *Zeit. wiss. Mik.*, vi, 1889, p. 508) gives several stains for tracheal cartilage, chiefly as microchemical tests, for which see *third edition.*

See also a critique of these methods by WOLTERS in *Arch. mik. Anat.* xxxvii, 1891, p. 492; and on the whole subject of cartilage see SCHIEFFERDECKER'S *Gewebelehre*, p. 331.

Fusari (*Arch. Ital. Biol.*, xxv, 1896, p. 200) makes sections of fresh cartilage, puts them for twenty-four hours into 1 per cent. nitrate of silver, washes, dehydrates, and exposes to the light in balsam.

See also Disse, *Anat. Anz.*, xxxv, 1909, p. 318, a stain for dentine, (hæmalum followed by a mixture of Saürerubin and Orange G); and Retterer and Lelièvre, *C. R. Soc. Biol.*, lxx, 1911, p. 630.

716. **Cartilaginous Skeletons** of embryos (Van Wijhe, *Proc. K. Akad. Wetensch. Amsterdam*, 1902, p. 47) may be studied by staining embryos for a week in a solution of 0·25 grm. methylen blue in 100 c.c. of 70 per cent. alcohol with 1 per cent. of hydrochloric acid. Wash out in alcohol with 1 per cent. of hydrochloric acid until no more colour comes away (about a week) and mount in balsam. The cartilage remains blue, all the other tissues being colourless.

Similarly, Lundvall (*Anat. Anz.*, xxv, 1904, p. 219, and xl, 1912, p. 639) using toluidin blue.

Similarly also Bakay (*Verh. Anat.Ges.*, 1902, p. 248), with Bismarck brown (the embryos having been previously treated with nitric acid of 3 per cent.).

CHAPTER XXX.

BLOOD AND GLANDS.

Blood.

717. Fixing and Preserving Methods.—The school of Ehrlich used to fix by *heat*. A film of blood was spread on a cover-glass and allowed to dry in the air, and then fixed by passing the cover a few times, three to ten or twenty, through a flame, or by laying it *face downwards* on a hot plate kept for several minutes or as much as two hours at a temperature at which water not only boils but assumes the spheroidal state (110° to 150° C.). For details see GULLAND, *Scottish Med. Journ.*, April, 1899, p. 312; RUBINSTEIN, *Zeit. wiss. Mik.*, xiv, 1898, p. 456; ZIELINA, *ibid.*, p. 463. But I believe they have now well-nigh abandoned this barbarous practice.

In *wet methods* either the blood is *mixed at once*, on being drawn, with some fixing and preserving medium, and studied as a fluid mount, or *films* are prepared and put into a fixing liquid *before they have had time to dry*; or after *drying in the air without heat* for a few seconds (at most 10 to 30).

To make a film, place a *very small* drop of blood on a *perfectly clean* slide. Bring down on to the slide the edge of another slide held over it at a slope; move this along till it touches the edge of the drop and the blood runs along the angle between the two slides. Then move the second slide *away* from the drop, and the blood will *follow* it and be drawn out into a film *without being crushed.* Similarly with two cover-glasses, to make a cover-glass film, which can be floated face down on to fixing or staining liquids in a watch-glass.

Some persons make films by flattening blood between two cover-glasses which are afterwards separated by sliding the one over the other; but that produces an injurious kneading of the cellular elements.

Most of the usual fixing agents are applicable to blood. But it is often necessary to employ only such as are favourable to certain stains. Those most recommended in this respect are alcohol, formol, sublimate (should not be too strong), osmic acid in very light fixation, or absolute methyl alcohol, which is an energetic fixative of dried films.

Air-dried films ought to be fixed before putting into aqueous or glycerin stains, else they will wash off; but this is not necessary for alcoholic stains.

718. Fixing and Preserving in Bulk.—Most morphologists are agreed that by far the most *faithful* fixing agent for blood-corpuscles is osmic acid. A drop or two of blood (BIONDI recommends two drops exactly) is mixed with 5 c.c. of osmic acid solution, and allowed to remain in it for from one to twenty-four hours. As a rule the osmic acid should be strong —1 to 2 per cent. Fixed specimens may be preserved for use in acetate of potash solution (MAX FLESCH, *Zeit. wiss. Mik.*, v, 1888, p. 83).

GRIESBACH also (*ibid.*, 1890, p. 328) combines the osmic acid with certain stains. He mentions methyl green, methyl violet, crystal violet, safranin, eosin, Säurefuchsin, rhodamin, and iodine in potassic iodide.

ROSSI (*ibid.*, vi, 1889, p. 475) advises a mixture of equal parts of 1 per cent. osmic acid, water, and strong solution of methyl green, permanent mounts being made by means of glycerin cautiously added.

EWALD (*Zeit. Biol.*, xxxiv, 1897, p. 257) mixes three to four drops of blood of amphibia or reptiles with 10 c.c. of a solution of 0·5 per cent. osmic acid in 0·5 per cent. salt solution (for mammals 0·6 to 0·7 per cent. salt), siphons off the supernatant liquid after twenty-four hours with his capillary siphon (§ 3, p. 4), and substitutes water, alum-carmine, etc., and lastly, 50 per cent. alcohol.

WEIDENREICH (*Arch. mik. Anat.*, lxxii, 1908, p. 213) lays a cover with a drop of blood on it on a layer of agar-agar (1 per cent. in salt solution of 0·8 per cent.) and after five minutes runs in osmic acid of 1 per cent., and after five minutes more removes the cover.

Dekhuysen (*Anat. Anz.*, xix, 1901, p. 536) recommends a mixture of either 3 or 9 vols. of 2 per cent. osmic acid with 1 of 6 per cent. acetic acid, containing $\frac{1}{8}$ per cent. of methylen blue, which he calls "Osmaect."

The mercurial liquids of Pacini (§ 414) used to be considered good. Hayem ("*Du Sang*," etc., Paris, 1889; see also *Zeit. wiss. Mik.*, vi, 1889, p. 335) has the following formula: sublimate 0·5, salt 1, sulphate of soda 5, and water 200. This should be mixed with blood in the proportion of about 1 : 100. Eosin may be added to it. Löwit's formula (*Sitzb. k. Akad. Wiss. Wien*, xcv, 1887, p. 144) consists of 5 c.c. cold saturated sublimate solution, 5 grms. sulphate of soda, 2 grms. salt, and 300 c.c. water. Mosso finds that both of these are too weak in sublimate.

Duboscq (*Arch. Zool. Expér.*, vi, 1899, p. 481) uses (for blood of Chilopoda) a solution of acetic acid, copper acetate, copper chloride, osmic acid, thionin, 1 grm. each, water 400, which, mixed with the blood, fixes and stains in about two minutes.

Formol has lately been used. Marcano (*Arch. de Med. Expér.*, xi, 1899, p. 434) mixes fresh blood with a mixture of 100 parts of sodium sulphate of sp. gr. 1·020 and 1 of formol; or with water 85 to 100 parts, sodium chloride 1, and formol 1.

Kizer (*Journ. Roy. Mic. Soc.*, 1900, p. 128) simply mixes 1 drop of blood with 3 of 2 per cent. formalin, and allows to stand for an hour.

Schridde (*Hæmat. Techn. Jena*, 1910, p. 17) lets blood drop into a mixture of 1 part of formol, 9 of liquid of Müller, and 10 of water, fixes therein for 2 to 4 hours at 40° C., filters, washes and brings through alcohol and chloroform into paraffin for sectioning.

719. Fixing and Preserving in films.—Muir (*Journ. of Anat. and Phys.*, xxvi, 1892) makes cover-glass films and drops them into saturated sublimate solution, and after half an hour washes, dehydrates, and passes through xylol into balsam.

Gulland (*Brit. Med. Journ.*, March 13th, 1897; *Scottish Med. Journ.*, April, 1899) makes cover-glass films, and after a few seconds drops them face downwards into a solution of—

Absolute alcohol saturated with eosin .	25 c.c.
Pure ether	25 „
Sublimate in absolute alcohol (2 grms. to 10 c.c.)	5 drops.

After three or four minutes they are washed, stained, and mounted in balsam.

For JENNER's fixing and staining and staining method, see next §.

Many recent authors fix wet films with *formol.* BENARIO (*Deut. med. Wochenschr.*, 1895, p. 572) mixes 1 part of 10 per cent. formol with 9 of alcohol (the mixture must be freshly prepared), and plunges films into it for a minute.

Similarly GULLAND, with 1 part of formol to 9 of alcohol.

Similarly WERMEL (see *Zeit. wiss. Mik.*, xvi, 1899, p. 50), who combines various stains (methylen blue, eosin, gentian, etc.) with the formol.

EDINGTON (*Brit. Med. Journ.*, 1900, p. 19) exposes films for 15 to 30 minutes to *vapour of formol* under a bell-jar.

SCOTT (*Journ. of Path. and Bacter.*, vii, 1900, p. 131) exposes films to the vapour for about 5 seconds and drops into absolute alcohol, and after 15 minutes stains and mounts.

A *short* exposure (30 seconds) to vapour of *osmium* has also been recommended.

720. Stains for Blood.—*Fresh* (unfixed) blood can be stained on the slide.

TOISON (*Journ. Sci. méd. de Lille*, fév., 1885; *Zeit. wiss. Mik.*, 1885, p. 398) recommends that it be mixed with the following fluid:

Distilled water . . .	160 c.c.
Glycerin (neutral, 30° Baumé) .	30 „
Pure sulphate of sodium . .	8 grammes.
Pure chloride of sodium . .	1 gramme.
Methyl violet 5 B . .	0·25 „

(The methyl violet is to be dissolved in the glycerin with one half of the water added to it; the two salts are to be dissolved in the other half of the water, and the two solutions are to be mixed and filtered.) This mixture stains leucocytes sharply, which facilitates enumeration.

Bizzozero and Torre (*Arch. Sci. Mediche*, 1880, p. 390) dilute a drop with normal salt solution containing a little *methyl violet*, which stains nuclei intensely, cytoplasm less intensely.

Similarly Giglio-Tos (*Zeit. wiss. Mik.*, 1898, p. 166), diluting with saturated solution of *neutral red* in salt solution, which stains hæmoglobigenous granules in five to ten minutes. This is also recommended by Ehrlich and Lazarus, see § 309.

Similarly also Ross (*Trans. Path. Soc.*, 1907, p. 117), using polychrome *methylen blue*.

Levaditi (*Journ. Phys. path. Gén.*, Paris, 1901, p. 425) allows solution of *Brillantkresylblau* in alcohol to dry on a slide, puts a drop of blood on the dried layer, and covers. Similarly Cesaris-Demel (*Arch. path. Anat.*, 1909, p. 92), with a mixture of this dye and *Sudan III;* and Nakanishi (*Centralb. Bakt.*, 1901, p. 98), with methylen blue BB.

Fixed films may be treated with the usual tissue stains, *eosin* being an important one, as it stains rose-red all parts of blood-cells that contain hæmoglobin. Ehrlich's acid hæmatoxylin, with 0·5 gr. of eosin dissolved in it, is a good general stain. Or, stain with hæmalum, and then with eosin (0·5 per cent. in alcohol or water).

Ehrlich's triacid, § 296, gives good general views, and demonstrates neutrophilous granules. His mixture for eosinophilous cells has been given, § 311.

Pappenheim's panoptic triacid (on sale by Grübler) is Ehrlich's triacid with methylen blue in place of the methyl green.

Chenzinski's mixture, which is good, has been given, § 313. Stain for six to twenty-four hours in a stove. This gives rise to precipitates. To avoid them (Willebrand, *Deutsch. med. Wochenschr.*, 1901, p. 57) you may make a mixture of equal parts of 0·5 per cent. solution of eosin in 70 per cent. alcohol and saturated solution of methylen blue in water, and add acetic acid of 1 per cent. drop by drop till the mixture begins to turn red, and filter before use. Or (Michaelis, *ibid.*, 1899, No. 30) make (*a*) a mixture of 20 parts 1 per cent. aqueous methylen blue with 20 of absolute alcohol, and (*b*) a mixture of 12 parts 1 per cent. aqueous eosin with

28 of acetone, and for staining mix equal parts of these and stain for half a minute to ten minutes.

Jenner (*Lancet*, 1899, No. 6, p. 370) mixes equal parts of 1·2 to 1·25 per cent. water-soluble eosin (Grübler's) and 1 per cent. methylen blue, filters after twenty-four hours, washes the precipitate on the filter, dries it, and dissolves it in 200 parts of *absolute* methyl alcohol (the solution can be had ready made from Grübler & Hollborn). (Or, simply mix 125 c.c. of 0·5 per cent. solution of the eosin in methyl alcohol with 100 c.c. of 0·5 per cent. solution of methylen blue.) Cover-glass films are floated on to this, in which they are *fixed and stained* in three minutes. Wash off the stain with *a little water* (not under the tap), dry, and mount in balsam. Erythrocytes red, all nuclei blue, parasites blue, but with unstained nuclei.

The methods of May and Grünwald are closely similar to this.

Assmann (*Münch. med. Wochenschr.*, 1906, No. 28; "Das eosinsaure Methylenblau," Leipzig, 1908, p. 35) treats fresh films for half a minute to three minutes in a Petri dish with a few drops of Jenner's solution (from Grübler & Hollborn), then pours on 20 c.c. of distilled water with five drops of $\frac{1}{10}$ per cent. solution of lithium carbonate, leaves for five minutes, rinses in distilled water, dries with blotting paper, and mounts in neutral balsam.

The foregoing mixtures give a stain—seemingly due to the formation of an eosinate of methylen *blue*—in which the nuclei of blood-cells are blue and their plasma red to violet. It was made out by Romanowsky (*St. Petersburger med. Wochenschr.*, 1891) that *under certain conditions* mixtures of these two dyes give a stain which is in some respects the inverse of this, blood-cells being stained in divers hues, according to their kinds, and any *protozoan parasites* that may be present showing *red* nuclei and *blue* plasma; which greatly facilitates their detection and diagnosis. This reaction appears to be due to the formation of an eosinate—*not* of methylen blue, but—of *Methylenazur*, § 377. The method, only vaguely indicated by Romanowsky, has undergone, at the hands of Ziemann, Zettnow, Nocht, Reuter, Michaelis, Ruge, Maurer, Leishman, Giemsa and others, numerous modifications which have culminated in the establishment of a process worked out by GIEMSA as perhaps the most trustworthy and efficient of "Romanowsky" stains. This is as follows:

GIEMSA'S **Azur-eosin** process. You start with a mixture of eosin with *methylenazur* (instead of methylen blue). This mixture is very troublesome to prepare, and is best obtained ready made from Grübler & Hollborn (their "Giemsa'sche Loesung für Romanowsky-faerbung"*). *Air-dried films* (*Deutsch. med. Wochenschr.*, 1907, No. 17) are fixed in alcohol or in methyl-alcohol (two to three minutes), and dried with blotting paper. They are treated for ten to fifteen minutes with a dilution of 1 drop of the stock mixture to 1 c.c. of water, washed under a tap, dried with blotting paper, and again dried in the air and mounted in balsam, or (preferably) preserved unmounted. All reagents, especially the balsam, must be strictly *free from acid.*

Wet films (*ibid.*, 1909, p. 1751) are treated as follows: Fix them for twelve to twenty-four hours in a mixture of 2 parts saturated aqueous solution of sublimate with 1 of absolute alcohol. Wash, and treat for five to ten minutes with a mixture of 2 parts of iodide of potassium, 100 of water, and 3 of Lugol's solution. Wash, and treat for ten minutes with 0·5 per cent. solution of sodium thiosulphate. Wash, and stain as above (changing the stain for fresh after half an hour), for one to twelve hours. Then pass through mixtures of acetone with first 5, then 30, then 50 parts per cent. of xylol into pure xylol, and mount in cedar oil. This process is applicable to *sections.*

Or (*ibid.*, 1910, p. 2476) a slide is placed in a Petri dish and covered with a mixture of *equal parts of methyl-alcohol and stock mixture.* After half a minute this is poured off and enough distilled water poured in to cover the slide, and the whole is rocked to mix the two. After three to five minutes, wash in running water, dry, and mount in cedar oil.

By any of these processes nuclei (red) are demonstrated not only in hæmatozoa, but in many bacteria, spirochætæ, coccidia, sarcosporidia, etc.

---

* To make this up from Grübler's powders, dissolve 3 grms. of Azur II-eosin and 8 decigrammes of Azur II, in 125 grms. of glycerin and 375 of methyl-alcohol.

See also, for paraffin sections, SCHUBERG, in *Deutsch. med. Wochenschr.*, xxxv, 1909, No. 48, or *Zeit. wiss. Mik.*, xxvii, 1910, p. 161, who passes through acetone and xylol into balsam.

The older Romanowsky stains published by the authors mentioned above, as also Laveran's "Bleu Borrel" seem to be superseded by Giemsa's.

LEISHMAN'S Romanowsky Stain (*Brit. med. Journ.*, March 16th and September 21st, 1901) is as follows: To a 1 per cent. solution of Grübler's medicinal methylen blue in water add 0·5 per cent. of sodium carbonate, heat to 65° C. for twelve hours and let stand for ten days. Then add an equal volume of 0·1 per cent. solution of Grübler's Eosin extra B, let stand for six to twelve hours, collect the resulting precipitate on a filter, wash it until the wash water comes off colourless, dry and powder. For staining, dissolve 0·15 grm. in 100 c.c. of pure methyl alcohol. Stain cover-glass films (air-dried) for five to ten minutes; flood the film with water for one minute, and examine, or dry (without heat) and mount in xylol balsam. Nuclei in shades of red, cytoplasm bluish, parasites blue with ruby red chromatin.

RAADT (*Münch. med. Wochenschr.*, 1911, No. 27; *Zeit. wiss. Mik.*, 1912, p. 236) obtains a Romanowsky stain of blood and parasites with JENNER'S solution. Films fixed with alcohol and ether are first stained for five to ten minutes in solution of one part methylenblau med. puriss. Hoechst, 0·5 part of lithium carbonate and 100 of water, kept for at least three weeks and diluted with 10 vols. of water. Rinse with water, dry with blotting paper, flood with JENNER'S solution diluted with 2 or 3 vols. of water, and stain for five to ten minutes. Wash, dry with blotting paper, and mount.

721. PAPPENHEIM (*Anat. Anz.*, xlii, 1912, p. 525) recommends the following for sections of *hæmopoietic tissues*, and also of kidney, liver, hypophysis, suprarenals, lung, intestinal epithelium and central nervous system. Fix in Orth's Formol-Müller, stain sections for twenty minutes in a stove in "aqueous diluted alcoholic" solution of MAY-GRÜNWALD or JENNER diluted with 8 vols. of water; after-stain for forty minutes in the stove in "aqueous GIEMSA solution (15 c.c. of water with 10 drops of glacial acetic acid)"; differentiate in 100 c.c. of water with 5 to 6 drops of acetic acid; wash, dry between blotting-paper; dehydrate in mixture of equal parts of acetone and absolute alcohol, and mount in

neutral balsam. The result is not a Romanowsky stain, but a pale methylen-blue-eosin stain.

See also WRIGHT, *Pub. Massachusetts Gen. Hosp.*, iii, 1910, p. 1, or *Journ. R. Micr. Soc.*, 1910, p. 783.

For the special technique of eosinophilous cells see MARTINOTTI in *Zeit. wiss. Mikr.*, xxvi, 1909, p. 4 (alphabetical bibliography of eight pages.)

722. Demonstration of Blood-plates of Bizzozero (KEMP, *Studies from the Biol. Lab. Johns Hopkins Univ.*, May, 1886, iii, No. 6 ; *Nature*, 1886, p. 132).—A somewhat large drop of blood is placed on a slide, and quickly washed with a small stream of normal salt solution. The blood-plates are not washed away, because they have the property of adhering to glass. They may be stained with solution of 0·02 per cent. of methyl violet or 1 : 3000 of gentian violet, in salt solution. To make permanent preparations of them, they should first be fixed, by putting a drop of osmic acid solution on the finger before pricking it.

They may also be stained in films, especially by the Romanowsky method. According to PAPPENHEIM ("Farbchemie," p. 107) *Wasserblau* is almost specific for them.

WRIGHT (*Journ. Morph.*, xxi, 1910, p. 274) studies them in tissues, after fixation with formol or sublimate (not Zenker) by staining with a modified Giemsa stain, and bringing through acetone and oil of turpentine into turpentine colophonium. Details *loc. cit.* or *Journ. Roy. mic. Soc.*, 1910, p. 783.

See also DEKHUYSEN, *Anat. Anz.*, xix, 1901. p. 533 ; KOPSCH, *Intern. Monatschr. Anat. Phys*, xxi, 1904, p. 314, and xxiii, 1906, p. 359 ; DEETJEN, *Zeit. phys. Chem.*, lxiii, 1909, p. 1.

723. WEIGERT'S **Fibrin Stain** (*Fortschr. d. Med.*, v, 1887, No. 8, p. 228).—Sections (alcohol material) are stained in a saturated solution of gentian or methyl violet in anilin water (§ 286). They are brought on to a slide and mopped up with blotting-paper, and a little Lugol's solution is poured on to them. After this has been allowed to act for a sufficient time they are mopped up with blotting-paper, and a drop of anilin is poured on to them. The anilin soon becomes dark, and is then changed for fresh once or twice. The anilin is then *thoroughly* removed by means of xylol, and a drop of balsam and a cover are added. This stain may be applied to celloidin sections without previous removal of the celloidin.

See also the modifications of this method by KROMAYER (§ 656); BENECKE (§ 690); UNNA (*Monatssch. prakt. Dermat.*, xx, 1895, p. 140); WOLFF (*Zeit. wiss. Mik.*, xv, 1899, p. 310); and one of another sort by KOCKEL, *Centralb. allg. Path.*, x, 1899).

Glands.

724. Mucin.—HOYER (*Arch. mik. Anat.*, xxxvi, 1890, p. 310) finds that the mucin of mucus cells and goblet cells stains with *basic* tar colours and with alum hæmatoxylin, but not with *acid* tar colours. He obtained his best results by means of thionin, and good ones with toluidin blue, both of these giving a metachromatic stain—tissues blue, mucin reddish—and also with methylen blue (which is particularly useful from its power of bringing out the merest traces of mucin), safranin, etc.

Tissues should be fixed for two to eight hours in 5 per cent. sublimate solution, and paraffin sections stained for five to fifteen minutes in a very dilute aqueous solution of the dye (two drops of saturated solution to 5 c.c. of water).

Hyaline cartilage, the jelly of Wharton, and the Mastzellen of Ehrlich give the same reactions with basic dyes as mucin does.

See also SUSSDORF, *Deutsche. Zeit. Thiermed.*, xiv, pp. 345, 349 (*Zeit. wiss. Mik.*, vi, 1889, p. 205); BIZZOZERO, *Atti. R. Accad. di Sci. di Torino,* 1889 to 1892 (reports in *Zeit. wiss. Mik.*, vii, 1890, p. 61; and ix, 1892, p. 219); also UNNA, *ibid.*, xiii, 1896, p. 42.

The safranin reaction is not obtained with all brands of the dye; that of Bindschedler and Busch, in Bâle, gives it, whilst safranin 0 of Grübler does not. UNNA employs chiefly polychrome methylen blue.

As regards the thionin stain, see HÁRI, *Arch. Mik. Anat.*, lviii, 1901, p. 678.

BRUNO (*Bull. Soc. Nat. Napoli,* 1905, p. 220) fixes and stains the skin of the frog in a mixture of 100 c.c. of formol of 1·25 per cent. with 8 c.c. of 1 per cent. solution of thionin. Mucus glands red.

Kultschizky (*Arch. mik. Anat.*, xlix, 1897, p. 8) fixes in his mixture (§ 57), and stains sections either in safranin with 2 per cent. acetic acid, or in a similar solution of *neutral red* (two to three days, washing out with alcohol).

Mayer (*Mitt. Zool. Stat. Neapel.*, xii, 1896, p. 303, or *last edition*) gives the following two formulæ for mixtures that stain *exclusively* mucus.

725. Mayer's **Mucicarmine** (*op. cit.*, last §).—One gramme of carmine, and 0·5 grm. of aluminium chloride with 2 c.c. of distilled water heated over a small flame for two minutes, and made up to 100 c.c. with 50 per cent. alcohol. This gives a stock solution, which is as a rule to be diluted for use tenfold with distilled or tap water.

726. Mayer's **Muchæmatein** (*ibid.*).—Hæmatein 0·2 grm., aluminium chloride 0·1 grm, glycerin 40 c.c., water 60 c.c. An *alcoholic* solution may be made by dissolving in 100 c.c. of 70 per cent. alcohol, with or without the addition of two drops of nitric acid.

727. **Mucicarminic Acid** (Rawitz, *Anat. Anz.*, xv, 1899, p. 439).—One gramme of carminic acid, 2 of aluminium chloride, and 100 c.c., of 50 per cent. alcohol.

728. **Goblet Cells.**—So far as these contain mucin they give the reactions above described, see Paneth, *Arch. mik. Anat.*, xxxi, 1888, p. 113 *et seq.*; List, *ibid.*, xxvii, 1886, p. 481; and Guyeisse, *C. R. Soc. Biol.*, 1907, p. 1212.

For intestinal epithelium, especially the cells of Paneth, see also Martin, *Unters. ueb. Oberflächen u. Drüsenepithel*, Leipzig, 1910; and Kull, *Arch. mik. Anat.*, lxxvii, 1911, p. 541 (sections stained with alum hæmatoxylin, treated for 20 to 30 seconds with tincture of iodine, stained a few minutes with Victoria blue, then with eosin).

729. **Salivary Glands.**—Solger (*Unters. z. Naturlehre d. Menschen*, xv, 5 and 6, pp. 2—15; *Festschr. f. Gegenbaur*, ii, 1896, p. 211) demonstrates the granules in serous cells and half-moons of the submaxillary gland by hardening in a 10 per cent. solution of formol, and then making sections and staining with hæmatoxylin of Delafield or of Ehrlich, the granules taking the stain.

Krause (*Arch. mik. Anat.*, xlv, 1895 p. 94) stains sections either with Heidenhain's iron hæmatoxylin or with Ehrlich-Biondi mixture or thionin. See also Krause, *ibid.*, xlix, 1897, p. 709; and Müller, *Zeit. wiss. Zool.*, 1898, p. 640.

Grand-Moursel and Tribondeau (*C. R. Soc. Biol.*, liii, 1901, p. 187) recommend for *pancreas* Nicolle's "thionine phéniquée," which stains the insulæ of Langerhans hardly at all, the rest strongly.

730. Gastric Glands.—Kolster (*Zeit. wiss. Mik.*, xii, 1895 p. 314) differentiates the two kinds of cells in stomach glands by over-staining with hæmatoxylin, washing out with alcohol containing 1 per cent. of HCl, blueing with alcohol containing 1 per cent. of ammonia, and, after washing, staining for one to five minutes in a weak solution of Säurefuchsin. Peptic cells blue, parietal cells red. Osmic material cannot be employed.

Cade (*Arch. Anat. Micr.*, iv, 1901, p. 4) stains material fixed with Bouin's picroformol in Victoria blue of 1 per cent.

R. and L. Monti (*Rich. Lab. Anat. Roma*, ix, 1902) demonstrate ducts and canaliculi of delomorphous cells by Golgi's bichromate and silver impregnation, especially with rejuvenated material (see Sacerdotti), leaving it for 5 or 6 days in half-saturated sulphate of copper, then for 24 hours in the osmic-bichromate mixture. You can imbed in paraffin (rapidly).

731. Intestine.—Bensley (*Amer. Journ. Anat.*, v, 1906 p. 323) stains sections of glands of Lieberkühn in a mixture of equal parts of saturated solutions of Orange G and Säurerubin, and then with toluidin blue, and mounts in balsam.

732. Liver.—Braus (*Denkschr. Med. Nat. Ges. Jena*, v, 1896, p. 307) demonstrates the *bile capillaries* by the rapid method of Golgi, hardening in a mixture of one part formol with three parts liquid of Müller or $\frac{1}{3}$ per cent. chromic acid.

Eppinger (*Beitr. path. Anat.*, xxxi, 1902, p. 230) studies them by means of a complicated modification of Weigert's neuroglia stain, and Ciechanowski (*Anat. Anz.*, xxi, 1902, p. 426) by means of Weigert's myelin stain (the 1885 method).

Oppel (*Anat. Anz.*, v, 1890, p. 144; vi, 1891, p. 168) puts pieces of liver or spleen (alcohol material) for twenty-four hours into a solution of neutral chromate of potash ($\frac{1}{2}$ to

10 per cent.), then for twenty-four hours into a $\frac{3}{4}$ per cent. solution of silver nitrate, washes, dehydrates, and cuts without imbedding. The lattice fibres are only stained near the surface, so that tangential sections must be made.

Similarly BERKLEY, *ibid.*, 1893, p. 772, fixing in picric acid, then in an osmium bichromate mixture, and then silvering.

See also RANVIER, *Journ. de Microgr.*, ix, x, 1885–6; IGACUSCHI, in *Arch. path. Anat.*, xcvii, p. 142, or *Zeit. wiss. Mik.*, 1885, p. 243 (gold process for study of fibrous networks); KUPFFER, *Sitzb. Ges. f. Morph.*, etc., München, Juli, 1889, or *Zeit. wiss. Mik.*, vi, 1889, p. 506; KRAUSE (*Arch. mik. Anat.*, xlii, 1893, p. 57); and TIMOFEJEW, *Anat. Anz*,, xxxv 1909, p. 296 (sections of frozen tissue stained with methylen blue).

733. Spleen.—For *lattice fibres*, see OPPEL, last §.

KULTSCHITZKY (*Arch. mik. Anat.*, xlvi, 1895, p. 675) studies the *musculature* in sections (of material from liquid of Müller) stained for a day or more in a solution of lakmoid in ether and mounted in balsam.

For *elastic fibres* he puts sections for half an hour or a day into a mixture of 800 parts 96 per cent. alcohol, 40 parts 1 per cent. solution of carbonate of potash, 2 parts Magdala red, and 1 part methylen blue.

For the *blood-vessels* he puts sections of Müller material for a few minutes into a solution of one or two parts of Säurerubin in 400 parts of 3 per cent. acetic acid, washes out in 2 per cent. acetic acid, and after-stains in a similar solution of helianthin or Wasserblau until the red only remains in the erythrocytes.

See also WHITING (*Trans. Roy. Soc.*, Edinburgh xxxviii, 1896, p. 311); SCHUMACHER (*Arch mik. Anat.*, lv, 1899, p. 151); WEIDENREICH (*ibid.*, lviii, 1901, p. 251).

734. Lymphatic Glands.—For lattice-fibres especially, see ROESSLE & YOSHIDA, *Beitr. path. Anat.*, xlv, 1909, p. 110, or *Zeit. wiss. mik.*, xxvi, 1909, p. 295. Sections stained with hæmatoxylin and eosin, or Weigert's iron hæmatoxylin, or Bielschowsky's neurofibril stain as applied by MARESCH, *loc. cit.*, § 691. The sections should not remain for more than 15 to 30 minutes in the oxide bath.

See also for the *thymus* some very complicated methods of Salkind, *Anat. Anz.*, xli, 1912, Nos. 6 and 7.

735. Kidney.—Sauer (*Arch. mik. Anat.*, xlvi, 1895, p. 110) finds that for the renal epithelium the best fixative is Carnoy's acetic alcohol with chloroform (three to five hours, washing out with absolute alcohol). A mixture of nine parts alcohol with one of nitric acid is also good, as is liquid of Perényi. He stains with iron hæmatoxylin, and after-stains in a very weak solution of Säurerubin in 90 per cent. alcohol, which stains the ciliary plateau. He macerates with iodised serum or one-third alcohol, staining afterwards with dahlia.

Arnold (*Anat. Anz.*, xxi, 1902, p. 417) employs *intra vitam* staining methods for the study of the granules of the epithelium cells. Sections of fresh kidney are cut with a Valentin's knife, and brought into a very dilute solution of neutral red, or methylen blue, in which the granules stain in a few minutes or hours. Or saturated solutions of the dyes, or of indigo carmine, may be injected subcutaneously during life, at intervals of fifteen to twenty minutes, and after two to five injections the organ may be excised and sections made and examined (see §§ 208 and 342 to 344).

CHAPTER XXXI.

NERVOUS SYSTEM—GENERAL METHODS.

736. Introduction.—Microscopical research into the structure of the nervous system pursues two ends. Either it is desired to elucidate the *internal* organisation of nerve-cells and nerve-fibres, the processes employed to this end forming a group of *cytological* methods, or it is desired to study the forms of nerve-cells, the distribution of the divers groups of nerve-cells in the grey matter, the connections that are formed by means of nerve-fibres between them, and to follow out the course of the tracts of fibres that enter into the constitution of the white matter of the cerebro-spinal axis, the processes employed forming a group of the *anatomical* methods of neurology.

A large proportion of the methods used in the study of nerve-tissue in *peripheral* organs having already been described in the chapters on "Methylen Blue," "Impregnation Methods," "Tegumentary Organs," and "Muscle and Tendon," the following chapters are chiefly devoted to methods for the study of the *central* nervous system.

For minute details concerning the dissection and hardening of the encephala of Man and the larger Vertebrates see MERCIER, *Les Coupes du Système Nerveux Central*, 1894, Paris, Rueff) ; DÉJERINE, *Anatomie des Centres Nerveux*, Paris, 1895 ; BEVAN LEWIS, *The Human Brain*, London, Churchill ; OBERSTEINER, *Anleitung biem Studium des Baues d. nervösen Centralorgane im gesunden u. kranken Zustande*, Leipzig, Toeplitz ; VAN WALSEM, *Verh. Akad. Wetensch. Amsterdam*, vii, 1899 ; BONVICINI, *Zeit. wiss. Mik.*, xxvi, 1909, p. 410 ; BAYON. *Die hist. Untersuchungensmethoden des Nervensystems*, Würzburg, 1905 ; and SPIELMEYER, *Technik d. mikro. Untersuchung d. Nervensystems*, Berlin, 1911, and VENDEROVIČS, *Anat. Anz.*, xxxix, 1911, p. 414.

FIXATION.

737. Fixation by Injection.—Fixation, in the proper sense of the word, is, of course, out of the question in the case of the human subject. But in the case of the lower animals it is possible to introduce fixing liquids into the living nerve-centres by means of injection, thus ensuring a much better penetration of the reagents than can be obtained by simple immersion.

GOLGI (*Arch. Ital. de Biologie,* t. vii, 1886, p. 30) injects 2·5 per cent. solution of bichromate of potash, through the carotid if he wishes to limit the hardening to the encephalon, or through the aorta if he desires to fix the spinal cord.

DE QUERVAIN (*Virchow's Archiv,* cxxxiii, 1893, p. 489) injects solution of Müller warmed to body heat. For dogs 300 to 400 c.c. are required, for cats one third to one half that quantity. After injection the whole organ is put into solution of Müller for some weeks.

(Further details in *fourth edition.*)

MANN (*Zeit. wiss. Mik.,* xi, 1894, p. 482) injects through the aorta. He first injects for about twenty seconds physiological salt solution warmed to 39° C. to wash out the capillaries, then saturated solution of corrosive sublimate, warmed to 39° C. After five minutes of injection the brain is removed and put for twelve hours into the same sublimate solution, after which it is either put for permanent preservation into 0·1 per cent. solution of sublimate, or is at once passed through alcohol for imbedding in paraffin.

See also § 741 (GEROTA) and STRONG (*New York Acad. of Sci.,* January 13th, 1896; *Anat. Anz.,* xi, 21, 1896, p. 655; *Journ. Comp. Neurol.,* xiii, 1903, p. 291); and McFARLAND (*Journ. App. Micr.,* ii, 1899, p. 541).

HARDENING.

738. Hardening by the Freezing Method.—The ether freezing method is to be preferred. The sections should be floated on to water, treated for a minute on the slide with 0·25 per cent. osmic acid solution, and stained or otherwise treated as desired. See §§ 182 and 183.

For a detailed description of these manipulations see BEVAN LEWIS'S *The Human Brain.* Also NAGEOTTE, *C. R. Soc. Biol.,* lxvii, 1909, p. 542, who finds that if the tissues are soaked for twenty-four hours before cutting in formol of 3 per cent. the formation of ice crystals is diminished.

739. Hardening by Reagents.—If large pieces of nerve-tissue are to be hardened, it is necessary to take special precautions in order to prevent them from becoming deformed by their own weight during the process. Spinal cord or small specimens of any region of the encephalon may be cut into slices of a few millimetres' thickness, laid out on cotton-wool, and brought on the wool into a vessel in which they may have the hardening liquid poured over them. Or, still better, the preparations may be *suspended* in the liquid, see § 34.

Another plan, which is good, is to add to the hardening liquid enough glycerin or salt to make the preparations *just* float.

If the preparations are placed on the bottom of the vessel, they should never be placed one on another.

If it be desired to harden voluminous organs without dividing them into portions, they should at least be incised as deeply as possible in the less important regions. It is perhaps better in general not to remove the membranes at first (except the dura mater), as they serve to give support to the tissues. The pia mater and arachnoid may be removed partially or entirely later on, when the hardening has already made some progress. With material intended for the Golgi impregnation it is well not to remove them at all.

The *spinal cord*, the *medulla oblongata*, and the *pons Varolii* may be hardened *in toto*. The dura mater should be removed at once, and the preparation hung up in a cylinder-glass with a weight attached to its lower end, in order to counteract the torsions of the tissues that may otherwise occur.

The *cerebrum* should have plugs of cotton-wool put into the fissure of Sylvius, and as far as possible between the convolutions. Unless there are special reasons to the contrary, the brain should be divided into two symmetrical halves by a sagittal cut passing through the median plane of the corpus callosum. BETZ recommends that after a few hours in the hardening liquid the pia mater should be removed wherever it is accessible, and the choroid plexuses also.

The *cerebellum* should be treated after the same manner.

The hardening action of most solutions is greatly enhanced by heat. Thus WEIGERT (*Centralb. med. Wiss.*, 1882, p. 819; *Zeit. wiss. Mik.*, 1884, p. 388) finds that at a temperature of from 30° to 40° C. preparations may be sufficiently hardened in solution of Müller in eight or ten days, and in solution of Erlicki in four days, whilst at the normal temperature two or three times as long would be required.

But it seems that this rapid hardening does not always give the best results. SAHLI is of opinion that it does not (see *Zeit. wiss. Mik.*, 1885, p. 3), and other authors are of the same opinion.

On the other hand, the slowness of the action of chromic salts at the normal temperature is such that decomposition may be set up before the hardening fluid has had time to do its work. For this reason voluminous preparations that are to be hardened in the slow way should be put away in a very cool place—best of all in an ice safe. A hemisphere will require eight or nine months for hardening in this way.

Very *large quantities* of liquid should be taken, and be changed, at first, every day for fresh.

Reagents (except osmic acid) should at first be taken as weak as is consistent with the preservation of the tissue, and be changed by degrees for stronger.

See also PFISTER in *Neurol. Centralb.*, xvii, 1898, p. 643 (*Zeit. wiss. Mik.*, xv, 1899, p. 494).

740. The Reagents to be employed.—Those most used are *formol*, and the *chromic salts.*

BURCHARDT (*La Cellule*, xii, 1897, p. 337) says that "according to the unanimous judgment of all investigators, the bichromates of potash and ammonia should not be employed for the *cytological* study of nerve-cells." NISSL (*Encycl. mik. Technik*, ii, p. 253) holds that, for this purpose, only alcohol, formol, sublimate, and occasionally nitric acid, are admissible. But this does not refer to hardening for purposes of *fibre-anatomy*, nor indeed in an absolute sense to cytological studies. It means that these are the reagents best fitted for producing a "Nervenzellenæquivalentbild," that is—a *standard* and *regularly obtainable* fixation, always amenable to certain current stains, of the whole of the various kinds of nerve-cells found in nervous centres. For fibre-anatomy he himself uses bichromate of potash.

741. Formol.—Formol gives much *better penetration* than the chromic salts, and even than alcohol, and allows of the most *various stains*, including silver neuro-fibril stains and the Golgi impregnation.

Several writers insist that for nervous tissue it should *not be acid*; but some prefer it acid (see "Retina"). For neuro-fibrils it certainly should be neutral. To neutralise, it is generally sufficient to make up the solutions with *tap-water*. It is *not likely to overharden*.

I use 1 part of formol to 9 of water (or 8 if the formol has been keep long).

Weigert (*Beit. Kenntn. norm. mensch. Neuroglia*, 1895, p. 1146) puts portions of material of not more than half a centimetre in thickness for four days into a "4 per cent. solution of formol" (meaning, presumably, 4 per cent. *formaldehyde*).

Marcus (quoted from Fish, see below) hardens spinal cord for two or four weeks in a ½ per cent. solution of "formalin," (meaning also probably *formaldehyde*), then in Müller's fluid for a week in an oven at 37° C.

Van Gieson (*Anat. Anz.*, x, 1895, p. 494) has used "solutions of formalin of 4, 6, and 10 per cent.," followed by 95 per cent. alcohol. Myelin was found to be well preserved.

Lachi (*cf. Zeit. wiss. Mik.*, xii, 1895, p. 32) has had good results with "20 per cent. solutions of formol."

Fish (*Proc. Amer. Mic. Soc.*, xvii, 1895, p. 319) recommends:

Water	2000 c.c.
Commercial formalin . . .	50 ,,
Sodium chloride	100 grms.
Zinc chloride	15 ,,

Brains should be left in this mixture for a week or ten days or more, then transferred to a solution of water 2000 c.c., formalin 50 c.c., in which they may remain indefinitely if the jar be kept tightly covered.

Parker and Floyd (*Anat. Anzeiger*, Bd. ix, 1895, p. 156) advise (for sheep's brains) a mixture of—

Alcohol 95 per cent. . . .	6 volumes
Formol 2 per cent. . . .	4 ,,

Brains may be kept for months in the mixture (*ibid.*, 1896, p. 568).

Flatau (*Anat. Anz.*, xiii, 1897, p. 323) finds that brain augments in weight slightly in 10 per cent. formol solution (spinal cord somewhat more); whilst in 1 per cent. solution it may increase as much as 24 per cent.

GEROTA (*Zeit. wiss. Mik.*, xiii, 1896, p. 314) puts human brains into a 5 or 10 per cent. solution of formol, and after twenty-four hours removes the pia and changes the liquid; this is also further done every five to seven days, and in one or two weeks the hardening is complete. In the case of fœtal brains of *Canis*, *Felis*, and *Homo*, he first injects the vascular system with a 10 to 15 per cent. solution of formol in 85 per cent. alcohol, and then brings the heads into the 5 to 10 per cent. watery solution; after one or two days he removes the brains from the skull and puts them back for fifteen to twenty days into the formol.

KADYI (*Poln. Arch. Biol. Med. Wiss.*, i, 1901, p. 80) takes 5 parts of formol, 100 of water, and 2 of bicarbonate of soda, for four to ten days.

HRDLICKA (*Proc. U. S. Nat. Mus.*, xxx, 1906, p. 304) takes 3 parts of formol, 25 to 45 of water, and 72 to 52 of alcohol of 95 per cent.

STRECKER (*Zeit. wiss. Mik.*, xxviii, 1911, p. 17) fixes small pieces for twenty-four to forty-eight hours in 1 part of formol of 10 or 20 per cent. with 1 part of Ehrlich-Biondi triacid mixture, and imbeds in paraffin, thus getting a stain at the same time as a fixation. Similarly with toluidin blue, fixing it with ammonium molybdate.

742. Chromic Salts.—That most used is the bichromate of potash.

The liquid of ERLICKI has a more rapid action than the other solutions of chromic salts. SAHLI, however (*loc. cit.*, § 739), after having studied the action of the usual solutions, concludes that the best hardening agent for fresh tissues is *pure bichromate of potash*, in 3 or 4 per cent. solution, the hardening being done in a cold place. He rejects the liquid of Erlicki on account of the precipitates it so frequently gives rise to (see § 54).

OBERSTEINER is of the same opinion, recommending pure bichromate for general hardening purposes; whilst for the study of the most delicate structural relations he recommends fixing in Fol's modification of Flemming's liquid (§ 42) for twenty-four hours, followed by washing with water and hardening in 80 per cent. alcohol.

In view of the slowness of penetration of chromic salts, it is often advisable to treat preparations for twenty-four hours or more with alcohol of 80 to 90 per cent., or formol, before putting them into the hardening liquid, or to add formol (say 3 per cent.) to it, in order to avoid maceration of the deeper layers of tissue.

Bichromate of potash should be taken at first of not more than 2 per cent. strength; this is then gradually raised to 3 or 4 per cent. for the cord and cerebrum, and as much as 5 per cent. for the cerebellum. OBERSTEINER begins with 1 per cent., and proceeds gradually during six to eight weeks to 2 or 3 per cent. (This is at the normal temperature; at a temperature of 35° to 45° C. one or two weeks will do.)

Bichromate of ammonia should be taken of half the strength recommended for bichromate of potash, or even weaker at first; it may be raised to as much as 5 per cent. for cerebellum towards the end of the hardening.

NISSL (*Encycl. Mik. Technik.*, ii, p. 245) takes (for rapid hardening) 100 parts of liquid of Müller, 3 of formol, and enough glycerin to make the tissues float—for a few days,—then pure Müller or bichromate of potash.

BETZ'S methods (*Arch. mik. Anat.*, 1873, p. 101).—Brain and spinal cord are first hardened, for some days or weeks, in 70 to 80 per cent. alcohol containing enough tincture of *iodine* to give it a light brown coloration. (As fast as the alcohol becomes colourless more iodine must be added.) Then definitely hardened in bichromate of potash, of 3 per cent. for spinal cord, medulla oblongata, and pons, 5 per cent. for cerebellum, and 4 per cent. for cerebrum.

Unduly neglected nowadays.

Cerebrum (BEVAN LEWIS, *The Human Brain*, p. 102).—Methylated spirit, twenty-four hours in a cool place. Müller's solution, three days in a cool place. Then change the liquid; and after three days more substitute a 2 per cent. solution of potassium bichromate. At the end of the second week a solution of double the strength may be added; and if at the termination of the third week the mass is still pliable, and of the consistence of ordinary rubber, it is as yet unfit for section-cutting, and the reagent should be replaced by a solution of chromic acid.

Brain (HAMILTON, *Journ. of Anat. and Physiol.*, 1878, p. 254).—Put into—

Müller's fluid	3 parts
Methylated spirit	1 part

in an ice-safe. Change the solution in a fortnight or three weeks; or if it is found that the reagent has duly penetrated, remove to—

Bichromate of ammonia	1 grm.
Water	400 c.c.,

for one week. Then change the solution to one of 1 per cent. for one week, and 2 per cent. for another week, or longer. The pieces may be kept permanently in solution of chloral hydrate, twelve grains to the ounce.

Entire Encephalon (DEECKE, *Journ. Roy. Mic. Soc.*, 1883, p. 419).—Bichromate of ammonia in $\frac{1}{2}$ to 1 per cent. solution, according to the consistence of the brain. If it is soft he adds, say, $\frac{1}{6}$ to $\frac{1}{10}$ per cent. of chromic acid to the solution, and always $\frac{1}{6}$ to $\frac{1}{4}$ of the whole volume of alcohol.

Encephalon (M. DUVAL, ROBIN'S *Journal de l'Anatomie*, 1876, p. 497).—Bichromate of potash 25, water 1000; change after twenty-four hours, and again after three or four days. After two or three weeks place in chromic acid of 3 per 1000, change every day for the first week and after that every eight days until the middle of the second month. The preparations must remain at least two months in the chromic acid.

ORTH (*Berlin. klin. Wochenschr.*, 1896, No. 13) takes Formol-Müller (§ 113), changed every few days. Small pieces may be sufficiently hardened in a few days in a stove. This is now very popular.

BONVICINI (*Zeit. wiss. Mik.*, xxvi, 1909 p. 412) puts entire brains into 10 per cent. formol (first injected through the carotids or into the ventricles) for six to eight days, then slices of it (in the dark) into 4 parts bichromate of potash and 2·5 parts *sulphate of chromium* in 100 of water, changed weekly for two months (hemispheres), or twelve to fourteen days (medulla and pons), or five to six days (cord).

RAWITZ (*ibid.*, p. 338) puts formol material for exactly five days into alcohol with 10 per cent. of *tinctura iodi* P.G., then for eight to ten into saturated solution of bichromate, changed after the first day, then into 95 per cent. alcohol in the dark for three days.

743. OTHER REAGENTS.—*Osmic acid* is hardly useful for specimens of more than 2 or 3 millimetres' thickness. These, at a strength of 1 per cent., it will harden well in five to ten days.

Chromic acid is not much used *alone*. Its action is rapid, but uneven, and causes shrinkage and brittleness. A very little (say one to two drops of 1 per cent. solution for each ounce) added to bichromate solution will do no harm and will quicken the hardening.

Nitric acid has been, and still is, employed in strengths of 10 to 12 per cent., and gives particularly tough preparations.

Neutral acetate of lead in 10 per cent. solution affords an excellent preservation of ganglion cells, according to ANNA KOTLAREWSKI (see *Zeit. wiss. Mik.*, iv, 1887, p. 387).

TRZEBINSKI (*Virchow's Arch.*, 1887, p. 1; *Zeit. wiss. Mik.*, iv, 1887, p. 497) finds that ganglion cells (of the spinal cord of the rabbit and dog) are best preserved by hardening for eight days in 7 per cent. solution of *corrosive sublimate*, followed by alcohol containing 0·5 per cent. of iodine. Similarly, DIOMIDOFF (*ibid.*, p. 499), with brain. This process produces artificial "pigment spots"; they may be dissolved out by prolonged treatment with warm water, or in five minutes by strong solution of LUGOL.

FISH (*The Wilder Quarter-Century Book*, 1893, p. 335) and DONALDSON (*Journ. of Morphol.*, ix, 1894, p. 123) have found that bichromate of potash produces a slight increase both in weight and volume of brains of sheep, whereas all the other reagents tried produce a diminution of both these factors.

Several observers have lately been using *acetic alcohol*. So TIMOFEEW, *Intern. Monatsschr. Anat. u. Phys.*, xv, 1898, p. 259 (CARNOY'S second formula, § 85).

MANN (*Methods*, etc., p. 95), for cell-studies, puts for twenty-four hours into solution of 5 parts of iodide of potassium and 25 of iodine in 100 of water, then into 70 per cent. alcohol.

FISH (*The Wilder Quarter-Century Book*, 1893, p. 393) takes

Water	400 c.c.
95 per cent. alcohol	400 ,,
Glycerin	250 ,,
Zinc chloride	20 grms.
Sodium chloride	20 grms.

for about three days, then transfers for a week or more to a mixture of equal parts of the fluid and 70 per cent. alcohol, and finally stores in 90 per cent. alcohol.

OHLMACHER recommends his sublimate mixture, § 65, KODIS (*Arch. mik. Anat.*, lix, 1901, p. 212) fixes in saturated solution of *cyanide of mercury*, brings into 10 per cent. formol, and sections by the freezing method.

NELIS (*Bull. Acad. Sc. Belg.*, 1899, 1900, p. 726) fixes *spinal ganglia* for twenty-four hours in a solution of 20 grms. sulphate of copper, and sublimate to saturation, in a litre of 7 per cent. formol with 5 c.c. of acetic acid.

KING (*Anat. Rec.*, iv, 1910, p. 213) after trying over twenty-five methods on brains of fifty rats, concludes that the best is Ohlmacher's. The brain should be put into it for two to three hours, then for one into alcohol of 85 per cent., then 70 per cent. with iodine for at least twenty-four hours, then passed through ether into 2 per cent. celloidin for two to three days, and passed through chloroform and benzol into paraffin. Bouin's is the best of the formol liquids; Tellyesnicky's is the only one of the bichromate mixtures that equals it. All sublimate mixtures fix nuclei well, but vacuolise cytoplasm.

The reader will note that these results do not allow for subsequent impregnation operations.

744. Nervous Centres of Reptiles, Fishes, and Amphibia.—MASON (*Central Nervous System of Certain Reptiles*, etc.; WHITMAN'S *Methods*, p. 196) recommends iodised alcohol, six to twelve hours; then 3 per cent. bichromate, changed once a fortnight until the hardening is sufficient (six to ten weeks).

BURCKHARDT (*Das Centralnervensystem von Protopterus*, Berlin, 1892; *Zeit. wiss. Mik.*, ix, 1893, p. 347) recommends a liquid composed of 300 parts of 1 per cent. chromic acid, 10 parts of 2 per cent. osmic acid, and 10 parts of concentrated nitric acid, in which brains of Protopterus are hardened in twenty-four to forty-eight hours.

FISH (*Journ. of Morphol.*, x, 1, 1895, p. 234) employed for *Desmognathus* a mixture of 100 c.c. of 50 per cent. alcohol, 5 c.c. of glacial acetic acid, 5 grms. of corrosive sublimate, and 1 grm. of picric acid, fixing for twelve to twenty-four hours, and passing through the usual alcohols.

STRONG (*Journ. comp. Neurol.*, xiii, 1903, p. 296) fixes (and decalcifies at the same time) the heads of young *Acanthias* in a mixture of 9 parts of 5 per cent. iron alum and 1 part of formol, for about two weeks, makes paraffin sections, stains with hæmatoxylin, and differentiates in iron alum of 1 to 2 per cent.

SECTIONS.

745. IMBEDDING is by no means always necessary. Sections can be obtained from any part of the central nervous system without it. The material should be well hardened, and glued on to a piece of wood or cork by means of a rather thick solution of gum arabic. As soon as it begins to stick to the support the whole is thrown into 80 per cent. alcohol to harden the joint, after which it may be fixed in the object-holder of the microtome and cut.

Or, you may simply make a clean cut at the bottom of the specimen, dry it with blotting paper, and stick it on with sealing wax.

To cut, the knife should be wetted with alcohol or water.

If the latter, add a little soap to prevent it from running into drops on the knife.

If the collodion method has been taken it may be found that notwithstanding every precaution the collodion has not thoroughly penetrated the tissues. Good sections may, however, still be obtained by DUVAL's method of collodionising the sections. The cut surface of the tissue is dried by blowing on it, and is covered with a thin layer of collodion laid on it with a brush. As soon as this layer has somewhat dried, which happens very rapidly, a section is cut and the cut surface is collodionised as before, and so on for each section. This process gives very good results, and may be advantageously employed even with material that has been successfully imbedded, as it gives a better consistency to the tissue, and enables thinner sections to be obtained (VAN GEHUCHTEN, *in litt.*).

STRASSER (*Zeit. wiss. Mik.*, ix, 1892, p. 8) obtains paraffin sections of 10 cm. breadth by 15 cm. length. He cuts out from hardened material slices of from 1 to 2 cm. in thickness, de-alcoholises them with xylol-carbolic acid mixture, § 167, allows this to evaporate, and brings them first into melted yellow vaselin, and lastly either into a mixture of vaselin and paraffin of 42° melting point, or into pure paraffin.

See also POSO, *ib.*, xxvii, 1910, p. 353 ; DEECKE, *loc. cit.*, § 742 ; DÉJERINE, *Anat. Centres Nerveux*, p. 29.

STRASSER also imbeds the slices in celloidin, and clears them before cutting with a mixture of xylol-carbolic acid and 80 per cent. alcohol in equal parts.

FEIST (*Zeit. wiss. Mik.*. viii, 1892, p. 492) marks the right and left sides of spinal cord by imbedding with each segment of it a small cylinder (of about 1 square millimetre in section) of hardened liver, stuck vertically in the imbedding mass (either celloidin or paraffin) against the side of the cord that it is desired to mark.

For the *freezing method* see p. 117, and for further details concerning imbedding and cutting see *fourth edition*.

LIESEGANG (*Zeit. wiss. Mik.*, xxvii, 1910, p. 369) *mounts* large sections, direct from water, in a layer of 5 per cent. solution of gelatin, lets this dry, and varnishes it, dispensing with balsam and cover.

GENERAL STAINS.

746. Carmines.—*Ammonia-carmine* is good for general views. Stain very slowly in extremely dilute solutions. Bichromate material ought to be brought *direct into the stain without passing through alcohol* (see § 51).

Picro-carmine has much the same action, but gives a better demonstration of non-nervous elements.

I prefer *carmalum,* with formol material, as giving a more delicate stain. I find it better than paracarmine.

Recent authors recommend *soda-carmine.* The *Encycl. mik. Technik.,* p. 927, advises staining Müller material for a couple of days in a 2 per cent. solution of carminate of soda (Grübler's).

BORAX-CARMINE, with indigo-carmine or an anilin blue to follow, gives elegant but not very instructive images, and I have abandoned it.

See also SCHMAUS (*Münch. med Wochenschr.,* 1891, No. 8; *Zeit. wiss. Mik.,* viii, 1891, p. 230); UPSON (*Neurolog. Centralb.,* 1888, p. 319; *Zeit. wiss. Mik.,* v, 1888, p. 525); FREEBORN (*Amer. Mon. Mic. Journ.,* 1888, p. 231; *Journ. Roy. Mic. Soc.,* 1889, p. 305); KADYI, *Neurol. Centralb.,* xx, 1901, p. 687; *Zeit. wiss. Mik.,* xviii, 1902, p. 483); CHILESOTTI (*ibid,* xix, 1902, p. 161, and xx, 1903, p. 87); KAPPERS and KETJEN (*ibid.* xxviii, 1911, p. 275) (Weigert material after-stained with paracarmine).

747. Anilin blue-black has been much recommended by SANKEY (*Quart. Journ. Mic. Sci.,* 1876, p. 69); BEVAN LEWIS (*Human Brain,* p. 125); VEJAS (*Arch. f. Psychiatric,* xvi, p. 200); GIERKE (*Zeit. wiss. Mik.,* 1884, p. 376); MARTINOTTI (*ibid.,* p. 478); JELGERSMA (*Zeit. wiss. mik.,* 1886, p. 39); SCHMAUS (*Münch. med. Wochenschr.,* No. 8, 1891. p. 147; *Zeit. wiss. Mik.,* viii, 1891, p. 230), and others. As to this colour see § 329, and for details see *previous editions.*

748.—Nigrosin has given useful results in some hands. I have not succeeded, probably because the dye is of inconstant composition, and does not keep well.

749. Picronigrosin.—MARTINOTTI (*loc. cit.,* 1884, p. 478) stains for two or three hours or days in a saturated solution of nigrosin in saturated solution of picric acid in alcohol, and washes out in a mixture of 1 part of formic acid with 2 parts of alcohol.

JOHNSTON (*Morph Jahrb.,* xxxiv, 1905, p. 150) adds a little Säurefuchsin to the mixture.

750. KAISER (*Zeit. wiss. Mik.,* vi, 1889, p. 471) stains sections of spinal cord for a few hours in a solution of 1 part of naphthylamin brown, 200 of water, and 100 of alcohol, washes with alcohol, clears with origanum oil, and mounts.

751. **Alizarin.**—Schrötter (*Neurol. Centralb.*, xxi, 1902, p. 338; *Zeit. wiss. Mik.*, xix, 1903, p. 381) stains sections for twenty-four hours in a 1 to 2 per cent. solution of sulphalizarinate of soda, differentiates for $\frac{1}{2}$ to 1 minute in tap-water, dehydrates, and mounts. This is a general stain, but demonstrates Nissl bodies and other internal details.

752. Nissl's *methylen-blue* is used as a general stain by some.

Röthig (*Folia Neurobiol.*, ii, 1909, p. 385; *Zeit. wiss. Mik.*, xxvi, 1909, p. 282) fixes and stains for about four weeks in saturated solution of *Methylenazur* I (Grübler) in formol of 10 per cent., puts for ten to fifteen minutes into aceton, then for twelve hours into chloroform, and imbeds in paraffin. He also has a process with trichloracetate of lead and methylenazur.

Rawitz (*Zeit. wiss. Mik.*, xxvi, 1910, p. 341) has some complicated methods with *Indulin*, *Indamin blue*, and *Azosäureblau*, which take twenty-eight days; and (*ibid.*, xxviii, 1911, p. 1) others with *fuchsin* and *azofuchsin* which take over thirty-six days.

Kappers (*ibid.*, p. 417) describes a stain of chromic material with *extract of elderberries.*

Scarpatetti (*Neurol. Centralb.*, xvi, 1897, p. 211; *Zeit. wiss. Mik.*, xiv, 1897, p. 91) stains sections of formol material for five minutes in 1 per cent. hæmatoxylin, treats for five minutes with concentrated solution of neutral acetate of copper, differentiates with Weigert's borax-ferri-cyanide, treats with concentrated solution of carbonate of lithia, washes and mounts. Myelin is not stained.

753. Mallory's **Phospho-molybdic-acid Hæmatoxylin** has been given, § 271.

For the extremely complicated modification of Auerbach, see *Neurol. Centralb.*, xvi, 1897, p. 439, or *Zeit. wiss. Mik.*, xiv, 1897, p. 402, and for that of Kodis see § 271.

754. **Hæmatoxylin and Säurefuchsin.**—Finotti (*Virchow's Arch.*, cxliii, 1896, p. 133; *Zeit. wiss. Mik.*, xiii, 1896, p. 236) stains in hæmatoxylin, counter-stains for three minutes with 0·5 to 1 per cent. solution of Säurefuchsin, and differentiates in 75 per cent. alcohol containing a very little caustic potash.

Van Gieson's hæmatoxylin and picro-Säurefuchsin, § 398, gives useful general views of nerve-cells, axis-cylinders, and neuroglia.

755. Alt (*Münch. med. Wochenschr.*, 1892, No. 4; *Zeit. wiss. Mik.*, ix, 1, 1892, p. 81) stains for a couple of hours in solution of *Congo red* in absolute alcohol, and washes out with pure alcohol. For peripheral axis-cylinders, and other elements.

CHAPTER XXXII.

NERVOUS SYSTEM—CYTOLOGICAL METHODS.

756. Introduction.—The ordinary methods of cytology are of course available for nerve-cells. But there are two characteristic elements of these cells—the tigroid substance, and the system of neuro-fibrils, which require, for minute study, special methods such as the following.

A. *Methods for Cells, demonstrating Tigroid Substance.*

757. Tigroid substance is a markedly basophilous element, occurring in the form of granules or larger irregular blocks known as the "bodies of Nissl." It takes up basic anilin dyes, but does not hold them with such special energy, as for example, the chromatin of nuclei. It is usually stained by the regressive method, with very careful differentiation. The material is usually fixed with alcohol, formol, or sublimate. Van Gehuchten and Nelis (*La Cellule*, xiv, 1898, p. 374) recommend Gilson's mixture, § 69.

All the following stains have the defect of *keeping badly*; they generally do not last more than a few months.

758. Nissl's **Methylen-blue Method** (*Neurol. Centralb.*, 1894, p. 508).—Fresh material is hardened in 96 *per cent. alcohol*, and sectioned *without imbedding*. The sections are *floated* on to the following stain poured into a watch-glass :

Methylen blue (Methylenblau B. pat.) .	3·75	parts.
Venice soap	1·75	,,
Distilled water	1000·0	,,

(This stain should not be used fresh, but kept for at least three months.)

The watch-glass is warmed over a flame to about 65° to 70° C. till bubbles are given off. The sections are then brought for an instant (5 to 20 seconds) into a mixture of

10 parts of anilin oil with 90 parts of 96 per cent. alcohol, and as soon as no more colour is given off from them are got on to a slide, dried with filter-paper, cleared with oil of cajeput, dried again with filter-paper, treated with a few drops of benzin, and mounted.

Van Gehuchten (*in litt.*) prefers to take *paraffin sections*, mounted on slides by the water method, and stain them for five or six hours in Nissl's mixture in a stove kept at 35° to 40° C.

Rehm (*Münch. med. Wochenschr.*, 1892, No. 13; *Zeit. wiss. Mik.*, ix, 1893, p. 387) stains for half a minute to a minute in a *hot* 0·1 per cent. of methylen blue, washes in 96 per cent. alcohol till no more colour comes away, clears with origanum oil, and mounts in balsam.

Gothard (*C. R. Soc. Biol.*, v, 1898, p. 530) stains *celloidin sections* for twenty-four hours, *without heat*, in Unna's polychromatic methylen blue and differentiates in a mixture of 5 parts of creosote, 4 of oil of cajeput, 5 of xylol, and 16 of absolute alcohol.

Luithlen and Sorgo (*Neurol. Centralb.*, xvii, 1898, p. 640) differentiate in Unna's glycerin-ether mixture (§ 702), remove this with absolute alcohol, and clear in origanum oil.

Lennhoff (*ibid.*, 1910, p. 1) recommends polychrome methylen blue for 2 minutes, followed by Grübler's "Karbol-Methylgrün-Pyronin" for 20 minutes.

Lord (*Journ. Ment. Sci.*, October, 1898) makes sections of *fresh tissue, frozen*, treats them for a few seconds with a mixture of equal parts of 6 per cent. formaldehyde and saturated solution of picric acid, then rinses, and warms till bubbles appear in 5 per cent. solution of methylen blue.

Mentz von Krogh (*Centralb. Bakt.*, lviii, 1911, p. 95) stains paraffin sections for 5 minutes in polychrome methylen blue, treats for 1 to 15 with 2 per cent. chromic acid, differentiates till blue with 5 per cent. tannin, and mounts in balsam. Shows also axis cylinders.

See also Goldscheider & Flatau, *Normale und. path. Anat. der Nervenzellen*, etc., Berlin, Kornfeld, 1898 (*Zeit. wiss. Mik.*, xvi, 1899, p. 102), and Nissl's remarks thereon, *Deutsche Zeit. Nervenheilk.*, xiii, 1899, p. 318 (*Zeit. wiss. Mik.*, xvi, 1899, p. 370); Cox, *Intern. Monatsschr. Anat. Phys.*, xv, 1898, Heft 8; Myers, *Anat. Record*, 1908, p. 434;

Ilberg, *Neurol. Centralb.*, 1896, No. 18; Savini, *Centralb. Bakt.*, 1909, p. 697.

759. Methylen Blue and Erythrosin.—Held (*Anat. Phys., Anat. Abth.*, 1895, 1896, p. 399) stains sections on slides, with the aid of a gentle heat, for one or two minutes in a solution of 1 grm. of Grübler's erythrosin in 150 of water with two drops of glacial acetic acid, washes out with water, and stains in a mixture of equal parts of Nissl's methylen blue and 5 per cent solution of acetone, warming strongly the while, until all odour of acetone has disappeared. After cooling he differentiates with 0·1 per cent. solution of alum until the sections appear reddish, rinses in water, dehydrates as rapidly as possible in absolute alcohol, and passes through xylol into balsam. See further hereon Held, *op. cit.*, 1897, pp. 226—233, 273—305 (Supplementband), and Boccardi, *Mon. Zool. Ital.*, x, 1899, p. 141 (stains in a mixture of erythrosin 0·1, toluidin blue, 0·2, and water 100 parts, and differentiates in 0·5 per cent. alum solution).

760. Thionin.—Lenhossék (*Fein. Bau. d. Nervensystems*, Berlin, 1894, p. 149) stains sections of *formol material* for five minutes in a concentrated aqueous solution of thionin, rinses with water, and mounts as Nissl. The stain does not keep well.

Similarly Ramón y Cajal, *Man. de Anat. Path. Gen.*, 1896 (see *Zeit. wiss Mik.*, xv, 1899, p. 375), and Luxenburg, *Neurol. Centralb.*, xviii, 1899, p. 629.

761. Toluidin Blue.—Lenhossék (*Neurol. Centralb.*, xvii, 1898, p. 577; *Zeit. wiss. Mik.*, xv, 1899, p. 492). Sections are stained on slides for a night in concentrated solution of toluidin blue, rinsed in water, quickly differentiated with alcohol, cleared with xylol or carbolic-acid xylol, and mounted in balsam.

Similarly Polumordwinow (*Zeit. wiss. Mik.*, xvi, 1899, p. 371, who stains in a very weak alkaline solution, 1 part of 1 per cent. solution to 119 of water and 1 of carbonate of soda.

762. Neutral Red.—Juliusburger (*Neurol. Centralb.*, xvi, 1897, p. 259) stains sections of formol material, for half to three quarters of a minute in *warm* 1 per cent. solution of neutral red, dehydrates in alcohol, and passes through bergamot oil to balsam.

Rosin (*Deutsche med. Wochenschr.*, 1898, No. 39, p. 615; *Zeit. wiss. Mik.*, xvi, 1899, p. 238) stains in concentrated aqueous solution, washes out thoroughly with water, and passes through alcohol (must be free from acid) into xylol and balsam. Granules of Nissl red, nucleoli red, all the rest yellow.

763. Alizarin.—See § 751.

764. Cresyl Violet. — Bielschowsky & Plien (*Neurol. Centralb.*, 1900, p. 1141) stain for 24 hours in 50 c.c. of water, with 6 to 10 drops of concentrated aqueous solution of Cresyl violet RR. and pass through water, alcohol, cajeput oil and xylol into balsam. The preparations *keep better* than thionin or toluidin blue ones.

765. Tigroid Substance, other Methods.—See Cox, *Zeit. wiss. Mik.*, xiii, 1896, p. 498; xv, p. 369; xvi, 1899, p. 101; *Anat. Hefte*, xxxi, 1898, p. 75; *Intern. Monatsschr.*, xv, 1898, H. 8; Auerbach, *Monatsschr. Psychiatrie*, iv, 1898, p. 31; *Zeit. wiss. Mik.*, xv, 1899, p. 493; Buehler, *Verh. Phys-Med. Ges. Würzburg*, xxxi, 1898, p. 285; *Zeit. wiss. Mik.*, xv, 1899, p. 351; Mosse, *Arch. mikr. Anat.*, 1902, p. 403 (his *argentamin* stain, which see).

B. *Methods for Cells and Fibres, demonstrating Neurofibrils.*

766. Neurofibrils; General Characters.—Nerve-cells, and the fibres into which they are prolonged, contain, in addition to the chromatic, basophilous element demonstrated by the method of Nissl, a characteristic "achromatic" element, consisting chiefly of fine, fairly refractive fibrils, which can only be seen with difficulty in the unstained state, and can only be well brought out by means of special stains. They may be fixed with *osmic acid*, and made out in thin sections of medullated nerves studied in dilute glycerin or water, and may be, to a certain extent, isolated by maceration.

Kupffer (*Sitzb. math. Kl. Akad. wiss. München*, xiii, 1884, p. 470) proceeded as follows: A medullated nerve is stretched on a cork and treated for twenty-four hours with 0·5 per cent. osmic acid. It is then washed in water for two hours and stained for twenty-four to twenty-eight hours in saturated aqueous solution of Säurefuchsin; after which it is washed out for from six to twelve hours (not more in any case) in absolute alcohol, cleared in clove oil imbedded in paraffin, and cut. It is said that if sections are mounted by the water method the stain will be extracted; but why not make the water *acid*, § 291?

The usual histological stains either leave them colourless or stain the surrounding plasma more strongly than the fibrils themselves. There may thus be produced a "negative" image of fibrils which does not really show the true neurofibrils, and being taken for them may prove a source of error. The following methods are such as have been recommended as giving true stains of the fibrils.

767. Neurofibrils: Silver Methods.—Those most used are RAMÓN Y CAJAL'S and BIELSCHOWSKY'S. The essential difference between the two is that Ramón employed a *single* impregnation bath—of nitrate of silver; whilst Bielschowsky employs two—one of nitrate and one of silver oxide dissolved in ammonia.

Fixing agents should *in general* be *neutral* or *alkaline*; for acids or oxidants divert the impregnation from the fibrils to other elements, chiefly tigroid and nuclear elements.

Sections should be *thin*—not more than 15 μ to 20 μ.

Toning with gold has the effect of intensifying the stain in the fibrils and lightening it in surrounding elements, thus giving enhanced contrast. It also serves to favour the preservation of the stain.

The object of fixing with hyposulphite is to remove from the tissues any unreduced silver salts which might cause a loss of contrast by darkening the ground of the preparations. With well reduced preparations, such as Ramón's are in general, it is not necessary.

Bielschowsky's methods have the advantage of being applicable to *larger specimens* than Ramón's, for they give a more uniform impregnation through the whole thickness of the objects (especially with the central nervous system of man); whilst Ramón's (especially his formula 1a) only gives the desired results in a—sometimes very thin—layer between an overstained outer and an understained inner one.

Bielschowsky's are applicable to *very old* formol material. BAYON (*Die histologischen Untersuchungs-Methoden des Nervensystems*, p. 157) has succeeded with material four years old. But this must be about the limit, for I find material seven years old entirely refractory.

The two methods do not give quite the same images of neurofibrils. Ramón's tends to show intra-cellular fibrils anastomosing into networks, whilst Bielschowsky's (like Bethe's) tends rather to show independent fibrils traversing cells without anastomosing.

For the demonstration of pericellular networks, buds of Held and Auerbach, non-medullated fibres and nerve-endings in general Ramón's seems the better.

Bielschowsky's method stains elastic fibres and connective tissue fibres, which Ramón's do not.

768. RAMÓN Y CAJAL'S **Methods.**—This section contains all the methods described by Ramón in *Trabajos del Lab. de Investigaciones Biologicas*, viii, 1910 (t. xiii of *Rev. trimestral micrografica*). The numbering is that of Ramón.

Formula 1a.—For small and medium nerve-cells. *Small* pieces of fresh tissue are put direct (*i. e.* without previous treatment with any other reagent) into nitrate of silver of 1·5 per cent.

They are kept for about three days (2½ for very small objects, such as spinal cord of newborn rabbit; 4 for medium-sized, such as cord or cerebellum of adult rabbit; 5 for specimens of the size of its cerebrum) in the silver. They must be kept in a stove at a temperature of about 35° C. all the time. (In summer, with a temperature constantly over 22° C., the stove may be dispensed with, provided that the impregnation be prolonged for two or three days more.) The tissues are known to be ripe for reduction when a freshly cut surface shows a brownish-yellow coloration.

They are then washed for one or two minutes in distilled water, and put into—

Pyrogallol or hydroquinon . .	1 to 2 grm.
Water	100 ,,
Formol*	5 ,,

* The formol is not necessary, but is useful. You may take pyridin instead (1 to 3 per cent.). You may also add 0·5 per cent. of sodium sulphite. The stronger the pyrogallol the greater the contrast: it is sometimes useful to take as much as 3 per cent., but then the over-impregnation of the outer layers will be increased. Hydroquinon (I add) reduces more energetically than pyrogallol.

They remain in this for twenty-four hours. They are then washed, hardened in alcohol, imbedded in colloidin or paraffin, and sections mounted in damar.

TELLYESNICKY (*Verh. Anat. Ges.*, 1904, p. 183) advises toning the sections for five to thirty minutes in 150 c.c. of water with 4 c.c. of 1 per cent. gold chloride. This is good for weak impregnations, but not desirable for strong ones which show good contrast.

Sections from the outer layers are too dark for study, those from the innermost too pale (if the specimens are large ones), whilst those from intermediate layers are fit for study. The over-stain of the outer layers can be diminished by diluting the silver bath with 1 volume of water for the last twelve hours.

This method has the defect of giving an imperfect fixation and impregnating almost exclusively cell-bodies and dendrites. It is not good for the large cells of adults, but excellent for small and medium cells of newborn or very young subjects, and for very early embryos in general.

Formula 1a A.—As last, but with nitrate of silver of 3 to 6 per cent.

DOGIEL (*Anat. Anz.*, xxv, 1904, p. 558) finds this method gives results not attainable by other means in the study of the corpuscles of GRANDRY (stoving for four to six days). Similarly KOLMER (*ibid.*, xxvi, 1905, p. 560) with epiderm of *Lumbricus*, etc.; and other authors for the ganglionic cord of Hirudinea.

Formula 1a B.—As before, but nitrate bath of only 0·75 per cent., and very small pieces of tissue, preferably embryos and newborn subjects. Poor fixation, much shrinkage, but vigorous stain of neurofibrils, of nucleolar granules, and of the *intra-nuclear rodlet* of RONCORONI.

Formula 1a C.—As before, but silvering in nitrate of 2 per cent. with one fourth of *absolute alcohol* or *acetone* added. Better fixation than pure nitrate. Gives results very similar to those of 1a with dog, cat and rabbit, and better results with human cerebrum and cerebellum.

Formula 2a.—*Fixation* for 24 hours in alcohol of 96 per cent. Tissues not washed, but mopped with blotting-paper, and put into nitrate of silver of 1·5 per cent. for seven days at 35° C., or six days at 40° C. The rest as

Formula 1a.* Good impregnations of nerve-centres of adults, of peripheral nerve-endings, of regenerating nerves, and of early embryos and of young fishes. Impregnates medullated (and many non-medullated) fibres (black), large and medium neurones (fibrils brown), the basket fibres of cells of Purkinje, the granular layer, and in the cerebrum large and medium pyramidal cells and nerve-fibres. Results fairly constant, but sometimes showing a granular precipitate of unknown origin.

To hinder this precipitate, and at the same time to hasten the impregnation, it is well to add to the alcohol certain substances which Ramón calls "accelerators." Such are chloral hydrate, veronal, pyridin, nicotin, ethylamin, antipyrin, and others.

Hypnotics, particularly veronal and chloral, and in a less degree pyridin and ammonia, also act as *rejuvenators*, reviving the susceptibility of impregnation which has been lost by tissues that have lain too long in alcohol. Ramón has thus succeeded with pieces of cerebrum and cerebellum that had been half a year in alcohol.

Formula 2a A.—Fixation for 24 to 48 hours in alcohol of 96 per cent. with 2 per cent. of *hydrate of chloral* added. Silver bath of 1·5 per cent. for five days in the stove. The rest as usual. *Veronal* (same proportion) gives the same result, as do also *sulphonal, trional, hedonal,* etc. The results are very constant. Medullated fibres well shown.

Formula 2a B.—Fix (time not stated) in alcohol with 10 to 20 per cent. of **pyridin,** wash for some hours in pure alcohol, and silver as usual (5 days). Results regular and constant.

Formula 2a C.—Fix for 24 hours in 50 c.c. of alcohol with ten drops of *nicotin.* Mop up with blotting paper, without washing, and silver as usual for five days (or four at 40° C.). Good results with adult tissues, especially spinal cord. Good penetration and less shrinkage than with pure alcohol.

---

* If the impregnation of inner layers should be too weak, the sections may be toned with—

Water	100 c.c.
Sulphocyanide of ammonium	3 gr.
Hyposulphite of sodium	3 ,,
1 per cent. gold chloride	a few drops.

Formula 2a D.—Fix for 24 hours in *allyl alcohol* (the industrial product will do). Wash for some hours in several changes of water. Put for a day into 50 c.c. of alcohol with 4 drops of ammonia. Silver for 4 days at 35° to 38° C., and reduce as usual. Good for human tissues, especially fibre plexuses of cerebrum and cerebellum. Instead of allyl alcohol you may take *acetal* or *aceton*. Put for six hours into aceton with 25 per cent. of water, then for 24 into pure aceton.

Formula 3a.—Fixation in *ammoniacal alcohol* for 20 to 48 hours. The most generally useful formula is, 50 c.c. of alcohol of 96 per cent. with four to five drops of ammonia (of 22° strength). But for cerebrum not more than one to three drops: for cerebellum, ganglia, spinal cord and regenerating tracts, four drops: for neurofibrils of the large neurons of the bulb and cord, nine to ten drops. To avoid shrinkage, it is well to begin by putting for six hours into alcohol of 70 per cent., then 85 per cent. without ammonia, then for the rest of the time into the ammoniacal alcohol. *Do not wash*, but mop up with blotting paper before putting into the silver. Silver for four to four and a half days (small specimens) at 40° C., or medium to large (3 to 4 mm. thick) for 5 days at 32° to 35° C. So long as the tissues are only yellowish-white, they are not ripe for reduction; light grey indicates ripeness; dark grey over-ripeness. Reduce as formula 1a.

Specimens *may be decalcified*, after reducing and washing, conveniently in alcohol of 96 per cent. with a few drops of nitric acid.

For delicate impregnation of fibrils of the soma of large and medium neurones, this formula is superior to all others. It gives good results with the majority of nerve centres, and is particularly good for non-medullated fibres, peri-cellular baskets of cerebellum, buds of Held and Auerbach in the oblongata, for human sympathetic, and for the study of regenerating elements.

Formula 3a A.—Fix in 50 c.c. of alcohol with 10 grm. of glycerin and six to ten drops of ammonia. Good for retina, non-medullated fibres, and especially the buds of Held and Auerbach.

Formula 3a B.—Fix in 50 c.c. of alcohol with 1·5 c.c. of

33 per cent. alcoholic solution of *ethylamine*. Results the same as with ammoniacal alcohol.

Formula 4a.—Pieces of tissue of not more than 4 mm. in thickness are fixed for six to twelve hours in *formol* of 15 per cent. Wash for six or more hours in running water.* Put for 24 hours into 50 c.c. of alcohol with five drops of ammonia. Wipe with blotting paper, silver for 5 days (or four if the stove is at 38° to 40° C.). The rest as usual. Sharp impregnation of the finer fibres of nerve centres, and of the terminal buds of pericellular nests. Adult tissues give better results than young ones. Energetic stain of the arborisations of the mossy fibres of the cerebellum.

Formula 4a A.—Fix in "a mixture of formol and alcohol." Wash out thoroughly with running water, and silver and reduce as usual. Fixation more rapid and better, results similar to those of 3*a*.

Formula 5a.—Small pieces put first for 6 to 8 hours into a mixture of equal parts of distilled water and *pyridin*, then for 18 to 24 hours into *pure pyridin*. Wash for several hours in running water, and put for a day into alcohol of 90 per cent. Wipe, and put for 4 to 5 days into the silver at 35° to 38° C., and reduce as usual. Not very good for adult organs, but superior to all others for the earliest phases of neurogenesis, and good for regenerative processes.

Formula 6a.—Put for 24 hours into 50 c.c. of water with 5 grms. of *hydrate of chloral*, rinse and put into 50 c.c. of alcohol of 96 per cent. with five drops of ammonia (time not stated). Wipe with blotting paper and put for 4 to 5 days at 35° to 38° C. into nitrate of silver of 1·5 per cent., and reduce as usual. Results very constant, without shrinkage. Stains perfectly the fine plexuses of cerebrum, bulb and cord, the Purkinje baskets and mossy fibres; also motor plates. Nuclei sufficiently stained to enable cells to be easily recognised. Good for regenerating nerves.

Formula 6a A.—Fix for 24 hours in *chloral of* 10 *per cent.*, wash for 6, and put *direct* into the silver. Stove four days. Results similar to those of 1a. Medullated fibres well stained.

---

* If formol (or pyridin or the like) be not thoroughly removed by washing, the stain will be weakened, and of a light reddish tone. (But I am not clear that this vigorous washing out is altogether advisable.)

Formula 7a.—Fix for 24 hours in Merck's *fibro-lysin*, wash for 6, put for 24 into 50 c.c. of alcohol with five drops of ammonia. The rest as usual.

Instead of fibrolysin, *lysidin* may be taken.

Application of the Foregoing Methods to Different Objects of Study.

(1) For the study of the evolution of neuroblasts and nerve fibres in *very early embryos*, it is absolutely necessary to avoid fixing with formol, or alcohol with an accelerator, or ammoniacal liquids. The best formulæ are 2a and 5a. Applicable to all vertebrates, but preferably to embryos of birds and fishes.

(2) For *late embryos* and *fœti* of mammals.—Besides the above, 3a, 6a and alcohol with an accelerator. Best subjects, embryos of chick from the fifth day, and of rabbit from the tenth to the twelfth. Or newborn birds, with ammonia alcohol or 5a.

(3) For *sympathetic ganglia.*—Formula 3a, or pure alcohols or 4a and 5a. Best with man. Dog, cat, and rabbit give mostly weak reactions. The visceral ganglia are the most difficult.

(4) *Sensory ganglia.*—Formula 2a, or 3a. Easy.

(5) *Cerebellum.*—The most favourable of all nerve centres. For Purkinje cells, 1a or 3a. For the baskets, climbing fibres, and medium and small dendrites, 2a, or its variants. For terminal rosettes and collaterals of mossy fibres, and for the plexuses of the granular layer, 4a, or sometimes 5a or 6a. For the stellate cells of the molecular layer, 2a and 3a, on the dog.

(6) *Cerebrum.*—In general, the same formulæ as for the cerebellum, especially 1a, for pyramids of young dogs and cats (of eight to twenty days). In formula 3a, the proportion of ammonia should be diminished. For fine plexuses, 4a, 5a, and 6a.

(7) *Spinal cord* and *bulb.*—All the formulæ are applicable. For neurofibrils of motor cells the best subject is the dog of four to fifteen days, with formula 3a, with a large dose of ammonia (ten drops). Also the alcoholic fixatives with an accelerator. For medullated fibres, large and small, 2a or

6a. For buds of Held and Auerbach, and for fine plexuses, 4a, 3a A, or 5a.

(8) *Ganglia of Invertebrates.*—For the medicinal leech (not for other leeches), 1a with 3 to 6 per cent. of nitrate. For *Hæmopis, Aulostomum, Pontobdella* and *Glossiphonia*, 2a or, better 3a, with not more than two to five drops of ammonia, and silver of 3 per cent., three to three and a half days.

For further details see SÁNCHEZ in *Trab. Lab. Invest. Biol. Madrid*, vii, 1909, p. 31, or *Zeit. wiss. Mik.*, xxvii, 1910, p. 392.

Lumbricus is generally refractory to Ramón's methods. BOULE (*Le Nevraxe*, x, 1909, p. 15) obtains good impregnations by *acidifying* the fixatives. He takes (A) formol of 25 per cent. with 5 per cent. of acetic acid, or (B) the same with 0·5 per cent. of ammonia added, or (C) 100 c.c. alcohol, 25 c.c. formol, 5 c.c. acetic acid and 0·5 c.c. ammonia. These results are confirmed by KOWALSKI, *La Cellule*, xxv, 1909, p. 292, who also gets impregnations by simply starving worms for several days, or exposing them to cold (–5° C.) for a quarter of an hour.

(9) *Regenerating nerve tissue.*—For nerves operated a month or more previously, 2a or 3a with not more than three drops of ammonia will stain equally the old and the new fibres. For nerves operated not more than two to ten days previously, 3a with 4 to 6 drops of ammonia, 5a with pyridin and 4a, also sometimes 6a. For regenerations in cord, cerebrum and cerebellum, 3a with three drops of ammonia, or 5a with pyridin, or pure alcohol.

769. Variants of RAMÓN'S Methods.—BESTA (*Riv. Path. Nerv. Ment.*, Firenze, xv, 1910, p. 333) fixes for 48 hours in *alcohol with 5 per cent. nitric acid*, neutralises in alcohol with ammonia, and silvers and reduces as Ramón.

KATÓ (*Folia Neurobiol.*, ii, 1908, No. 3; *Zeit. wiss. Mikr.*, xxvi, 1909, p. 281) fixes in formol of 10 to 15 per cent., and silvers for one to five days at 35° C. in 5 per cent. *argentamin* to which has been added nitrate of silver of 3 per cent. in excess, and reduces in 10 per cent. formol with 1 per cent. of hydroquinon.

PUSATERI (*Arch. Path. Anat.*, 195 Bd., 1909, p. 547) fixes for three to six days at 35° to 38° C. in a mixture of 45 c.c. of *tachiol* (10 per cent. solution of fluoride of silver) with 155 of water, rinses and reduces for 24 hours in formol of 5 to 10 per cent. with 1 to 2 per cent. of hydroquinon. He tones the paraffin sections in 10 c.c. of water with 2 drops of acetic acid and five of 1 per cent. gold chloride, and fixes with hyposulphite of soda of 5 per cent.

Liesegang (*Kolloidchemie*, Beihefte, iii, 1911, H. 7; *Zeit. wiss. Mik.*, xxviii, 1912, p. 369) makes *sections* of formol material by the *freezing process*, and silvers them until yellow. He then adds to the silver bath an equal volume of 50 per cent. solution of gum arabic and the same amount of saturated solution of hydroquinon. After one or two minutes the sections are brought into 10 per cent. solution of hyposulphite of sodium, washed and mounted. Results said to be the same as by the usual process.

770. Bielschowsky's **Methods** (Bielschowsky and Wolff, *Biol. Centralb.*, xxv, 1905, p. 683).—Objects of not more than 2 mm. in thickness are fixed in *neutral* formol of 6 to 10 per cent. (time not stated), washed out well with distilled water, and put for at least two days into nitrate of silver of 2 per cent. in the dark. Wash for a few minutes, and put for half an hour to several hours into a bath made as follows:—To nitrate of silver of 10 per cent. add drop by drop 40 per cent. solution of caustic soda until no further precipitate is formed (this will be about 5 drops to 10 c.c.), dissolve the precipitate almost entirely in just enough ammonia, filter and add 4 to 5 vols. of water. The solution will only keep for a few hours. After this bath, wash again, put for 1 to 6 hours into formol of 4 to 5 per cent., dehydrate and bring through xylol into paraffin. Sections are toned on the slide for 1 to 2 hours in chloride of gold of one tenth to one twentieth per cent. (which it is well to neutralise with lithium carbonate), rinsed, fixed for 5 to 15 minutes in hyposulphite of soda of 5 per cent., washed for 6 to 12 hours in running water and mounted in balsam.

If nuclei take the impregnation, the fibril stain will not have succeeded, but there may be a useful stain of other elements, especially tigroid matter.

Schlemmer (*Zeit. wiss. Mik.*, xxvii, 1910, p. 22) makes the oxide bath by adding the caustic soda in excess, and washing the precipitate by repeated decantations until the wash water no longer gives an alkaline reaction, takes it up with ammonia and filters through spun glass, and thus obtains a solution which will keep for several days. I find the filtering is not necessary.

For older forms of this method see *last ed.*, and *Neur. Centralb.*, xxiii, 1903, p. 977, and xxiv, 1904, p. 387 (the toning bath is here 10 c.c. of water with 2 to 3 drops of 1 per cent. gold chloride, and 2 to 3 of acetic acid.

Bielschowsky (*Journ Psych. Neurol.*, xii, 1909, p. 135; *Zeit. wiss. Mik.*, xxviii, 1911, p. 226) also has a method with *pyridin.* Formol material, up to one cubic centimetre for adult tissue, and up to 5 centimetres long for embryos, is put for 3 to 4 days into pyridin, washed for some hours in several changes of distilled water, and put for 3 to 5 days into nitrate of silver of 3 per cent. at a temperature of 36° C. It is then put for 24 hours into an oxide bath made as follows: Precipitate 5 c.c. of 20 per cent. solution of silver nitrate by 5 drops of caustic soda of 40 per cent., dissolve in ammonia, *q. s.*, add 100 c.c. of water (and, to hinder formation of precipitates in the tissues, a few drops of ammonia). After this bath, wash for a couple of hours in many changes of distilled water, and reduce in formol of 20 per cent., dehydrate and make paraffin sections. These may be toned, but there is not much gained by it.

He also (*ibid.*) makes sections by the *freezing method*, of formol material, and puts them for 24 to 48 hours into pyridin, and washes them out well with water as before. They are then put for 24 hours (or more) into 3 per cent. solution of nitrate of silver at the normal temperature; then into the oxide bath prepared as before, but with only 20 c.c. of water instead of 100, and without addition of ammonia (the bath ought not to contain an excess of ammonia recognisable by the smell). They remain in this until yellowish-brown, but not more than half an hour, are well washed, and reduced in formol of 20 per cent. They are then toned with gold and fixed with hyposulphite as described *ante.*

By these pyridin methods intra-cellular fibrils are generally not so well shown as by the older method, but axis cylinders come out better, and glia remains unstained. The method succeeds even with material that has been for years in acid formol, and gives a uniform impregnation of entire blocks f tissue.

Schütz (*Neurol. Zentralb.*, xxvii, 1908, p. 909) finds that the times given by Bielschowsky are too short—which is not at all my experience. For toning, he puts the sections for 10 minutes into 10 c.c. of water with two drops of acetic acid, then for 30 to 45 into 10 c.c. of water with three drop of 1 per cent. gold chloride (until blackish-grey). He fixes for 3 to 5 minutes in 10 c.c. of sodium hyposulphite of 5 per cent. with one drop of acid sodium sulphite, and washes out for 24 hours in distilled water.

PATON (*Mitth. Zool. Stat. Neapel,* xviii, 1907, p. 576) fixes fish embryos in 4 per cent. formol neutralised with carbonate of magnesia, makes both silver baths only ¾ to 1 per cent. strong, washes with weak acetic acid, and reduces in 10 c.c. of 1 per cent. hydroquinon with 1 c.c. of formol. After toning, he stains with 1 per cent. solution of eosin in absolute alcohol.

SAND (*C. R. Ass. Anat. Bruxelles,* 1910; *Bibliogr. anat. Supp.,* 1910, p. 128) gives the following as entirely certain for man, dog, cat and rabbit. Specimens of not more than 5 mm. in thickness are fixed for 48 hours in a freshly prepared mixture of 90 parts of acetone and 10 of nitric acid (change for fresh after half an hour and once again within 24 hours). Wash out for at least 6 hours in pure aceton, changed two or three times. Make paraffin sections, and silver for three days at about 37° C. in 20 per cent. solution of silver nitrate. Put for ten minutes into a mixture (at least three days old) of 1000 parts of water, 10 of acetate of sodium, 5 of gallic acid, and 3 of tannin (to be changed if it becomes turbid). Mount at once or tone until grey (five minutes) in 80 parts of water with 17 of 2 per cent. solution of sulphocyanide of ammonium and 3 of 2 per cent. solution of gold chloride, and fix for a few seconds in 5 per cent. solution of hyposulphite of soda. Neurofibrils grey-violet, shown in cells, dendrites and axones. Terminal buds of Held also clearly shown, and nothing else stained. You may after-stain in any way, even with Weigert's or Benda's neuroglia stain.

BOEKE (*Anat. Anz.,* xxxv, 1909, p. 136) says that Bielschowsky's method will succeed after many kinds of fixation, even that by picro-sulphuric acid. For embryos he finds the best is 10 parts of formol to 90 of 60 per cent. alcohol, the objects being washed out with pure formol of 10 to 12 per cent. until all the alcohol is removed, before silvering.

LUGARO (*Monit. Zool. Ital.,* xv, 1904, p. 353) has a highly complicated method with *collargol* (colloidal silver) which has not met with favour.

771. **Neurofibrils: Gold Methods.**—Apáthy's gold method, § 371, gives perhaps the sharpest stain which has yet been obtained—with certain *invertebrates*, but *only with these*: and even with these it is difficult and uncertain.

Joris (*Bull. Akad. Med. Belg.*, April 30th, 1904) gives the following as being certain. Material fixed by the usual methods is put for 8 to 12 hours into a 5 per cent. solution of ammonium molybdate, then imbedded in paraffin. The sections (fixed on slides by the water method) must be washed for many hours or days in water. They are then treated for about ten minutes with a 1·5 per cent. solution of *colloidal gold* in water, rinsed and mounted. The stain is permanent.

The colloidal gold used was obtained from the Chemische Fabrik Heyden, in Radebeul-Dresden. It will dissolve in about a day.

772. **Neurofibrils, Molybdenum-Toluidin Blue** (Bethe, *Zeit. wiss. Mik.*, xvii, 1900, p. 13).—Pieces of *central* nervous system (*of Vertebrates*) are fixed for twenty-four hours in nitric acid of from 3 per cent. to 7·5 per cent. strength, and brought direct into alcohol of 96 per cent. for a day or more. They are put for twelve to twenty-four hours into a mixture of one part of ammonia (of sp. gr. 0·95) with three of water and eight of 96 per cent. alcohol, then for six to twelve hours into pure alcohol; they are then put for twenty-four hours into a mixture of one part concentrated hydrochloric acid with three of water and eight to twelve of alcohol, then for ten to twelve into pure alcohol, and thence for not more than two to six hours into water. They are now mordanted for twenty-four hours in a 4 per cent. solution of ammonium molybdate, brought for 24 hours into alcohol, and imbedded in paraffin (*not* celloidin). Sections are seriated on albumen (*without water*), then passed through xylol and alcohol into water, and "differentiated"—by which the author means washed out—with water. About 1 to 1·5 c.c. of distilled water should be poured on to the slide so as to form over the sections a layer 1·5 to 2 mm. deep, and the slide is put for two to ten minutes into a stove heated to not more than 55° to 60° C. The sections are then rinsed several times with water, a solution of one part of toluidin blue in 3000 of

water is poured on to them, they are again stoved for ten minutes, rinsed with water, treated with 96 per cent. alcohol till no more colour comes away, and passed through absolute alcohol and xylol into xylol balsam.

The method is also applicable to invertebrates, for which other fixations than nitric acid are admissible, and the impregnation with the molybdate may be done on the sections instead of the uncut tissues. The results are not so certain as for vertebrates.

Lugaro (*Riv. Pat. Nerv. Ment.*, Firenze, x, 1905, p. 269) modifies this by fixing in nitric acid dissolved (to 1 per cent.) in *aceton*.

Donaggio (*Ann. Nevrol. Napoli*, 1904, p. 161) fixes pieces not more than 5 mm. thick for five to six days in *pyridin*, changed at least once, washes in water, and mordants for twenty-four hours in ammonium molybdate 4 grms., water 100, hydrochloric acid 4 drops. Wash, get into paraffin, treat sections on slide for one minute with water, stain for three to thirty minutes in a 1 : 10,000 solution of *thionin*, and mount; or, better, first treat again for fifteen to thirty minutes with molybdate solution.

Jäderholm (*Arch. mik. Anat.*, lxvii, 1905, p. 108) finds that the pyridin causes enormous shrinkage, and that the thionin agglutinates the fibrils.

Paravicini (*Boll. Mus. Z. Anat. Comp.*, Torino, xx, 1905, p. 514) fixes and mordants in the dark, and differentiates after staining with extremely weak hydrochloric acid.

See also Tomaselli, *Zeit. wiss. Mik.*, xxiii, 1907, p. 422, and Montanari, *ibid.*, xxviii, 1911, p. 22, who describes observations which seem to throw doubt on the objectivity of the network described by Donaggio.

773. **Neurofibrils, Apáthy's Hæmatein Method** (*Mitth. Zool. Stat. Neapel.*, xii, 1897, p. 712).—Material may be fixed with sublimate, liquid of Zenker, picro-sulphuric acid, or any mixture that is not inimical to staining with alum hæmatoxylin, and should be preserved in 90 per cent. alcohol. Portions are stained for at least forty-eight hours in the hæmatein solution 1 A, § 259, and are then washed for up to twenty-four hours in *absolutely pure* distilled water, preferably suspended therein. Before the stain has become washed out of the neurofibrils, it is fixed therein by putting the preparations for three to five hours into *spring* water, after which they are put back for not more than two hours into distilled water, dehydrated as rapidly as possible by hanging them up

in absolute alcohol, and imbedded (they must be protected from the light whilst in the chloroform through which they are passed into paraffin, or whilst in celloidin). Sections are made and mounted in a resin or in neutral glycerin.

This method has given splendid results with Hirudinea and some other *invertebrates*, but I believe has not yet been successful with vertebrates.

774. **Neurofibrils, Berlin Blue** (S. MEYER, *Anat. Anz.*, xx, 1902, p. 535).—Material is best fixed in 10 per cent. formol, then mordanted for eight to twenty days in ferrocyanide of potassium of 2½ per cent. (or this salt may be added to the fixative), then put for two to four days into iron alum of 10 per cent. Wash for several hours, imbed in paraffin, and mount sections in balsam. Besides neurofibrils, the sheath of Schwann and the constrictions of Ranvier are stained. Like the Golgi chrome-silver process, this method gives a *partial* impregnation, only certain elements taking on the stain.

775. The **Methylen blue** *intra vitam* method is important, see the processes of APÁTHY, DOGIEL, and BETHE in Chapter XVI.

776. The methods of COX for the fibrils of spinal-ganglion cells, *Zeit. wiss. Mik.*, xiii, 1896, p. 498, and *Anat. Hefte*, x, 1898, p. 98, seem to be definitively superseded.

777. **Golgi's** INTRA-CELLULAR NETWORK.—For the study of his *apparato reticolare interno*, GOLGI (*Verh. Anat. Ges.*, xiv, 1900, p. 174) uses a mixture due to VERATTI, consisting of—

5 per cent. bichromate . . .	30 parts
0·1 per cent. platinum chloride . .	30 ,,
1 per cent. osmic acid . .	15 to 30 ,,

and after hardening therein puts (for a time varying from a few hours to ten days) into a mixture of 1 part of bichromate of 3 or 4 per cent. with two of saturated solution of sulphate of copper, and thence into silver nitrate of 0·8 to 1 per cent. See further *Verh. Anat. Ges.*, xiv Vers., 1900, p. 174.

More recently (*Arch. Ital. Biol.*, xlix, 1908, p. 272; *Mon. Z. Ital.*, xix, 1908, p. 263) he fixes for six to eight hours in a mixture of equal parts of 20 per cent. formol, saturated solution of arsenious acid, and alcohol of 96 per cent., and then puts for one to three hours (or days) into nitrate of silver of 1 per cent. He then reduces in any developer, usually 20 grms.

hydroquinon, 5 grms. sodium sulphite, 50 c.c. formol of 20 per cent., and 1000 c.c. water. Wash and imbed, preferably in celloidin. Tone the sections until grey in 1000 c.c. water with 30 grms. each of hyposulphite of soda and sulphocyanide of ammonium and 10 per cent. of 1 per cent. gold chloride. It is well, though not necessary, to treat them first with a solution of 0·5 grm. of permanganate of potash and 1 grm. of sulphuric acid in 1000 of water, and then with 1 per cent. solution of oxalic acid, before mounting.

LEGENDRE (*Anat. Anz.*, xxxvi, 1910, p. 209) omits the toning and permanganate, and imbeds in paraffin.

Similarly, COLLIN et LUCIEN, *Bibliogr. Anat. Supp.*, 1909, p. 238.

SARAGNONE (*Patologica*, i, 1909, p. 536; *Journ. Roy. Micr. Soc.*, 1910, p. 256) silvers with a mixture of 30 c.c. of *tachiol* (10 per cent. solution of silver fluoride) with 100 of water.

BESTA (*Anat. Anz.*, xxxvi, 1910, p. 477) fixes for two days in 20 parts of formol with two of acetic aldehyde and 80 of water, washes for twenty-four hours in distilled water changed 7 or 8 times, and puts for two days into 4 per cent. solution of ammonium molybdate, makes paraffin sections, stains in a 1 : 1000 solution of *thionin*, differentiates in 3 parts of creosote to 1 of absolute alcohol, and passes through pure creosote and xylol into neutral balsam. Recommended for Purkinje cells and spinal ganglia of young subjects.

KOPSCH (*Sitzb. Acad. Wiss. Wien*, xl, 1902, p. 929; *Zeit. wiss. Mik.*, xx, 1904, p. 347) demonstrates it in spinal ganglion cells as follows; the ganglia are put for eight days (or exceptionally, a few more), into osmic acid of 2 per cent. and paraffin sections made. The network becomes quite black and is sharply demonstrated, unless the impregnation has been too prolonged, in which case the rest of the cell body becomes blackened also.

SJÖVALL (*Anat. Hefte*, xxx, 1906, p. 362) fixes in formol before treating with the osmic acid.

778. Medullary Sheath (Neuroceratin, etc.).—GOLGI (quoted from REZZONICO, *Arch. per le Sci. med.*, iv, 1879, p. 85) puts pieces of *spinal cord* into 2 per cent. solution of bichromate of potash, for eight to fifteen days in summer, or a month in winter. Wash, and put into 0·75 per cent. solution of nitrate of silver, for two or three days in summer, or eight or ten or more in winter. Pass through alcohol into oil of turpentine, tease therein, and mount in damar. Expose to sunlight therein for eight to ten days; or to diffused daylight for twenty to forty days. Demonstrates funnels and spirals.

For *peripheral* nerves, GOLGI (*ibid.*, p. 238) puts pieces into the bichromate for from four hours to at most two days, passing specimens at intervals into the silver, where they remain for twelve to twenty-four hours. Wash in several changes of alcohol, tease therein, and pass through oil of turpentine into damar. Reduce therein in direct sunlight for a few days or weeks. The preparations *keep well.*

He also (*ibid.*, p. 238) puts pieces into a mixture of 10 parts 2 per cent. bichromate with 2 of 1 per cent. osmic acid, and passes them on into the silver at intervals of three hours, after the first four, during twenty-four hours. The silver is of 0·5 per cent. strength, and the pieces remain in it for any time not less than eight hours. The rest as before. Results somewhat more precise, but the stain does not keep in damar. It is important that the nerve should *not have been stretched.*

SALA (*Verh. Anat. Ges.*, 1900, p. 176) employs the method of VERATTI for the intracellular network, last §.

See also concerning these methods MONDINO, *op. cit.*, viii, p. 45, and PETRONE, *Intern. Monatsschr. Anat.*, v, 1888.

GALLI (*Zeit. wiss. Mik.*, iii, 1886, p. 467) puts for eighteen to twenty days into solution of Müller, then stains for fifteen to twenty minutes in aqueous solution of China blue, washes out in alcohol, clears in essence of turpentine, and mounts in damar.

PLATNER (*Zeit. wiss. Mik.*, vi, 1889, p. 186) fixes for several days in a mixture of 1 part of Liq. Ferri Perchlor. (Ph. G., ed. 2) and 3 to 4 parts of water or alcohol, washes out well in water and stains for several days or weeks in a concentrated solution of "Echtgrün" in 75 per cent. alcohol. See also BEER, *Jahrb. Psychiatrie*, ii, 1893, 1 Heft.

COX (*Anat. Hefte*, i, 1898, p. 75) fixes nerves in osmic acid of 2 per cent. (rabbit) or 1 per cent. (frog), washes, dehydrates, clears with bergamot oil, and mounts in balsam. The bergamot oil dissolves out the myelin, and leaves the neurokeratin visible. It may be necessary to leave the nerves for forty-eight hours in the oil.

CORNING (*Anat. Anz.*, xvii, 1900, p. 309) studies the network in the ischiatic of the frog on sections of sublimate material strongly stained with iron hæmatoxylin.

See also KAPLAN (*Arch. Psychiatr.*, xxxv, 1902, p. 825;

Zeit. wiss. Mik., xix, 1903, p. 508)—sections stained with Säurefuchsin and differentiated by the method of PAL.

GEDOELST (*La Cellule*, v, 1889, p. 136) has the following: (*a*) A nerve is treated with *liquid of* PERÉNYI, either pure or with addition of a trace of osmic acid, and examined in glycerin. By this treatment the myelin loses its excessive refractivity and the network comes out clearly. (*b*) Silver nitrate. Good images, but uncertain. (*c*) Treatment with a mixture of osmic acid of 1 per cent. and absolute alcohol. The network comes out black.

CHAPTER XXXIII.

MYELIN STAINS.

779. Iron Hæmatoxylin.—I find the simplest way to make a myelin stain is to make paraffin sections of formol material and stain them with iron hæmatoxylin, exactly as for central corpuscles (say twelve to fourteen hours in the mordant, six in the hæmatoxylin, and about two minutes for the differentiation). Sections best not over 15 μ. You may after-stain the cells (which are only grey) with carmalum, but not for more than half an hour, or the hæmatoxylin will be attacked. The stain is not so æsthetic as Weigert's, but quite as sharp. Axis cylinders are not shown.

Similarly Regaud, *C. R. Acad. Sci.*, clxviii, 1909, p. 861, but adding a *chrome mordantage* either concurrently with the formol fixation, or subsequently.

Also Nageotte, *C. R. Soc. Biol*, lxvii, 1909, p. 542, with sections of formol material by the *freezing method;* Houser, *Journ. Comp. Neurol. and Psych.*, x, 1901, p. 65, and Brookover, *ibid.*, xx, 1910, No. 2; Spielmeyer, *Neurol. Zentralb.*, xxix, 1910, p. 348, and his *Technik. d. mikro. Untersuch. d. Nervensystems*, 1911, p. 87, with sections of 25 to 35 μ by the freezing method; Loyez, *C. R. Soc., Biol.*, lxix, 1910, p. 511, who differentiates first lightly, till the grey begins to come out, in the iron alum, then washes, and differentiates further in Weigert's borax ferricyanide; Gilbert, *Zeit. wiss. Mik.*, xxviii, 1911, p. 279, who mordants with iron alum, stains with *molybdic acid hæmatoxylin,* and differentiates with the borax ferricyanide; Stoelzner, *ibid.*, xxiii, 1906, p. 329, who mordants celloidin sections of formol material for five minutes in *Liq. ferri sesquichlorati,* stains in hæmatoxylin of 0·5 per cent., and differentiates in the mordant or in borax ferricyanide; and Kodis, *Arch. mik. Anat.*, lix, 1901, p. 211, who fixes for one or two days in saturated solution of cyanide of mercury, hardens in 10 per cent. formol, and stains sections of frozen material by Heidenhain's iron hæmatoxylin.

780. Weigert's **Methods.**—There have been in all three methods of Weigert—the 1884 method, the 1885 method, and the 1891 method.

The 1884 method (*Fortschr. d. Med.*, 1884, pp. 113, 190; *Zeit. wiss. Mik.*, 1884, pp. 290, 564), which depends on the formation of a chrome lake of hæmatoxylin, may be considered to be superseded. Not so the two others, which depend on the formation of a copper lake in addition to the chrome lake.

781. Weigert's **1885 Method** (*Fortschr. d. Med.*, 1885, p. 136; *Zeit. wiss. Mik.*, 1885, pp. 399, 484; *Ergebnisse der Anatomie*, vi, 1896 [1897], p. 10).—The tissues are to be hardened in bichromate of potash. Weigert takes (*Ergebnisse*, p. 10) a 5 per cent. solution, and if time is an object hardens in a stove. (Other bichromate mixtures will do, *e. g.* Müller's, Kultschizky's, Zenker's; Erlicki's is not to be recommended.) The tissues are "ripe" for staining when the hardening has been carried to a certain point. They are first (*Ergebnisse*, p. 13) yellow, without differentiation of the grey matter from the white; these are unripe. Later they show the grey matter light brown, the white matter dark brown; and these are ripe.

More lately (*ibid.*, p. 14) he added to the bichromate solution 2 per cent. of chrome alum or of fluoride of chromium, which hastens the hardening, so that small specimens become brown and ripe in four to five days, without stoving.

After hardening, the preparation is imbedded in celloidin (if desired; imbedding is not obligatory) and hardened in the usual way. The hardened block is put for one or two days, in an incubating stove, into saturated solution of neutral *acetate of copper* diluted with one volume of water. By this treatment the tissues become green and the celloidin bluish-green. The preparation may then be kept till wanted for sectioning in 80 per cent. alcohol.

Sections are made, well washed in water, and brought into a stain composed of—

Hæmatoxylin	0·75 to 1 part.
Alcohol	10 parts.
Water	90 „
Saturated solution of lithium carbonate	1 part.

They remain there for spinal cord, two hours; medullary layers of brain, two hours; cortical layers, twenty-four hours.

They are then again well washed with water, and brought into a decolorising solution composed of—

Borax	2·0 parts
Ferricyanide of potassium . .	2·5 „
Water	200·0 „

They remain there until complete differentiation of the nerves (half an hour to several hours), and are then *well washed* with water (running, or changed several times), dehydrated, and mounted in balsam. They may be previously stained, if desired, with alum-carmine for the demonstration of nuclei.

The method is applicable to the study of peripheral nerves as well as to nerve-centres, and also to the study of lymphatic glands, skin (see Schieffeedecker, *Anat. Anz.*, ii, 1887, p. 680), bile capillaries, and other objects.

The process is applicable to tissues that have been hardened in alcohol or in any other way, provided that they be put into a solution of a chromic salt until they become brown, before mordanting them in the copper solution.

It is not necessary that the mordanting be done in bulk. Max Flesch (*Zeit. wiss. Mik.*, iii, 1886, p. 50) prefers (following Lichtheim) to make the sections first, and mordant them separately.

Vassale (quoted from Bayon's *Hist. Untersuchungsmeth. d. Nervensystems,* Würzburg, 1905, p. 124) *first* stains the sections in 1 per cent. hæmatoxylin, for three to five minutes, then puts for three to five minutes into saturated solution of acetate of copper, and differentiates.

782. Weigert's **1891 Method** (*Deutsche med. Wochenschr.*, 42, 1891, p. 1184; *Zeit. wiss. Mik.*, viii, 1891, p. 392).—The material is to be hardened in bichromate and imbedded in celloidin (see last §). It is then (according to the latest form of the process, *Encycl. mik. Technik.*, 1903, p. 942; for the earlier form see *last ed.*) put for 24 hours in a stove into a solution of 2½ parts of fluoride of chromium, 5 of acetate of copper, and 5 of acetic acid in 100 of water.*

Sections are then made and stained for from four to twenty-four hours at the temperature of the room in a freshly prepared mixture of 9 vols. of (A) a mixture of 7 c.c. of saturated aqueous solution of carbonate of lithium with 93 c.c. of water, and 1 vol. of (B) a solution of 1 grm. of hæmatoxylin in 10 c.c. of alcohol (A and B may be kept in stock, but A must not be too old). The sections should be *loose* ones, and not thicker than 0·025 mm. They are then washed in several changes of water, and treated with 90 per cent. alcohol, followed by carbolic-acid-and-xylol mixture (for a short time only), or by a mixture of 2 parts of anilin oil with 1 of xylol, then pure xylol and xylol balsam (not chloroform balsam).

It was, however, found that preparations thus made, *without differentiation*, did not keep well, and WEIGERT (*Ergebnisse d. Anat.*, iii, 1894, p. 21) reverted to the practice of differentiating with the borax-ferricyanide mixture, last §.

Later still (*Encycl. mik. Technik.*, 1903, p. 942) he employed a stain composed of equal parts of (A) a mixture of 4 c.c. of the officinal *Liquor ferri sesquichlorati* with 96 of water, and (B) a mixture of 10 c.c. of 10 per cent. solution of hæmatoxylin in alcohol with 90 of 96 per cent. alcohol. The two must be mixed immediately before use, and the sections should remain in the stain over night or longer, then be rinsed and differentiated as usual. This has the advantage of demonstrating very fine fibres, and of giving a *colourless ground.*

For difficult objects the differentiating liquid may be diluted with water, and gives better results than dilute acetic or hydrochloric acid or the like, which were formerly recommended.

Formol material may (*Ergebnisse*, vi, 1897, p. 14) be employed if mordanted till brown (four or five days) in 5 per cent. solution of bichromate with 2 per cent. of chromium fluoride.

---

* Instead of the chromium fluoride, you may take chrome alum, as Weigert did at one time, and as some still do. But then you must *boil*, as directed for Weigert's **Neuroglia stain**, § 838.

P. Meyer (*Neurol. Zentralb.*, xxviii, 1909, p. 353; *Zeit. wiss. Mik.*, xxvi, 1909, p. 488) imbeds and cuts *before* putting into the copper fluid.

Modifications of Weigert's Method.

783. Pal's **Method** (*Wien. med. Jahrb.*, 1886; *Zeit. wiss. Mik.*, iv, 1887, p. 92; *Med. Jahrb.*, 1887, p. 589; *Zeit. wiss. Mik.*, 1888, p. 88).—You proceed at first as in Weigert's process, but *omitting the copper bath*, and you stain as in Weigert's process. After staining in the hæmatoxylin solution the sections are washed in water (if they are not stained of a deep blue a trace of lithium carbonate must be added to the water). They are then brought for *twenty to thirty seconds* into 0·25 per cent. solution of permanganate of potash, rinsed in water, and brought into a decolouring solution composed of—

Acid. Oxalic. pur.	1·0
Potassium Sulphite* (Kalium Sulfurosum [SO_3K_2])	1·0
Aq. Dest.	200·0

In *a few seconds* the grey substance of the sections is decolourised, the white matter remaining blue. The sections should now be well washed out, and may be double-stained with Magdala red or eosin, or (better) with picrocarmine or acetic-acid-carmine.

Pal's process gives brilliant results, the ground of the preparations being *totally colourless.* Weigert (*Ergebnisse*, vi, p. 21) considers that for very thick sections it is superior to his own. But it is not so safe for very fine fibres.

Marcus stains by the Pal method sections of material hardened in *formalin*, as described § 741.

Gudden (*Neurol. Centralb.*, xvi, 1897, p. 24) makes celloidin sections of material hardened in 5–10 per cent. formol followed by alcohol, treats them for ten hours with 0·55 per cent. chromic acid, rinses with water, and treats with 80 per cent. alcohol, then stains by the method of Pal, adding to the hæmatoxylin a few drops of dilute nitric acid (Minnich).

---

* Not "sulphide," as erroneously given in Mercier's *Les Coupes du Système Nerveux Central*, p. 190.

Tschernyschew and Karusin (*Zeit. wiss. Mik.*, xiii, 1896, p. 354), stains for twenty-four hours in the *hæmatoxylin* of Kultschitzky, next §.

So also Pavlow, (*ibid.*, xxi, 1904, p. 14, taking the permanganate twice as strong as Pal.

Kozowsky (*Neurol. Zentralb.*, xxiii, 1904, p. 1041) stains as Weigert, and differentiates the sections first with 1 per cent. permanganate, till the grey comes out brown, and finishes the differentiation with *Liq. ferri sesquichlorati.*

Pötter (*Zeit. wiss. Mik.*, xxvii, 1910, p. 238) stains as Weigert, last §, and differentiates first in permanganate of 0·25 per cent., then in borax ferricyanide.

784. Kaiser (*Neurol. Centralb.*, xii, 1893, pp. 364, 368; *Zeit. wiss. Mik.*, xi, 1894, p. 249) hardens first in liquid of Müller, then for eight days in *liquid of* Marchi (§ 796), mordants sections for five minutes with sesquichloride of iron (1 part to 1 of water and 3 of 70 per cent. alcohol), stains, and differentiates with Pal's liquid. For details see *early editions.*

Bolton (*Journ. of Anat. and Phys.*, xxxii, 1898, p. 245) makes sections of formalin material, and mordants them for a few minutes in 1 per cent. osmic acid, or for a few hours in iron-alum or ammonium molybdate, stains in Kultschitzky's hæmatoxylin (next §), and differentiates by Pal's process.

Similarly Wynn, *ibid.*, 1900, p. 381.

Laslett (*Lancet*, 1898, p. 321; *Journ. Roy. Mic. Soc.*, 1898, p. 600) mordants in liquid of Marchi (1 week), makes sections, stains by Kultschitzky's method, and differentiates by Pal's.

785. Kultschitzky's method (*Anat. Anz.*, 1889, p. 223, and 1890, p. 519).—Specimens are hardened for one or two months in *solution of Erlicki*, imbedded in celloidin or photoxylin, and cut. Sections are stained for from one to three hours, or as much as twenty-four, in a stain made by adding 1 grm. of hæmatoxylin dissolved in a little alcohol to 100 c.c. of 2 per cent. *acetic acid.* They are washed out in saturated solution of carbonate of lithia or soda.

Differentiation is not necessary, but by adding to the carbonate of lithia solution 10 per cent. of a 1 per cent. solution of red prussiate of potash, and decolorising therein for two or three hours or more, a sharper stain is obtained. After this the sections are well washed in water and mounted in balsam. Myelin dark blue.

Wolters (*Zeit. wiss. Mik.*, vii, 1891, p. 466) proceeds as Kultschitzky, except that he stains at 45° C. for twenty-four hours, after which the sections are dipped in solution of Müller, and differentiated by the method of Pal.

Similarly KAES (*ibid.*, viii, 1891, p. 388 ; *Neurol. Centralb.*, 1891, No. 15). Myelin dark blue, cells yellow-brown.

786. MITROPHANOW (*Zeit. wiss. Mik.*, xiii, 1896, p. 361) mordants photoxylin sections for at least twenty-four hours at 40° C. in a mixture of equal parts of saturated aqueous solution of acetate of copper and 90 per cent. alcohol, stains for ten minutes in Kultschitzky's hæmatoxylin, and differentiates with Weigert's ferricyanide.

787. BERKLEY'S **Rapid Method** (*Neurol. Centralb.*, xi, 9, 1892, p. 270 ; *Zeit. wiss. Mik.*, x, 1893, p. 370).—Slices of tissue of not more than two and a half millimetres in thickness are hardened for twenty-four to thirty hours in *mixture of Flemming*, at a temperature of 25° C., then in absolute alcohol, then imbedded in celloidin and cut. After washing in water the sections are put overnight into a saturated solution of acetate of copper (or simply warmed therein to 35° to 40° C. for half an hour). They are then washed, and stained for fifteen to twenty minutes in a lithium carbonate hæmatoxylin similar to Weigert's, warmed to 40° C., allowed to cool, and differentiated for one to three minutes in Weigert's ferricyanide liquid, which may be diluted if desired with one third of water.

788. HILL (*Brain*, 1896, p. 1 ; *Phil Trans.*, 184, B, 1894, p. 399) stains well-washed Müller material in bulk in *alum carmine*, cuts, mordants for twenty-four hours in half-saturated acetate of copper, stains and differentiates as Weigert, taking the differentiating fluid only half as strong.

789. BENDA'S **Rapid Method** (*Berlin. klin. Wochenschr.*, No. 32, 1903).—Sections of *formol material* by the freezing process (alcohol being avoided) are stained (without any mordanting) for twenty-four hours in Boehmer's hæmatoxylin, differentiated with Weigert's ferricyanide, and mounted in balsam. Only recommended for peripheral nerves, or for preliminary examination of the central nervous system.

Similarly, NAGEOTTE, *C. R. Soc. Biol.*, 1908, p. 408, staining with hæmalum.

Similarly the *Encycl. mik. Technik.*, 1910, ii, p. 239, with *fresh material* cut by the freezing process, and the sections mounted in levulose (as alcohol somewhat extracts the stain).

790. Streeter (*Arch. Mik. Anat.*, lxii, 1903, p. 734) stains *small nerve-centres* in bulk (after mordanting in Weigert's bichromate and fluoride mixture, § 781) with Weigert's hæmatoxylin (four to six days), washes for a couple of days in 70 per cent. alcohol, makes *paraffin sections*, and differentiates them by the method of Weigert or Pal.

791. Besta (*Zeit. wiss. Mik.*, xxiv, 1907, p. 185) mordants nerves for one to three days in 100 c.c. of water with 25 of formol and 4 grms. of Merck's *ammonio-chloride of tin*, cuts in paraffin, stains in Mallory's hæmatoxylin and differentiates in solution of iodine in iodide of potassium.

792. **Gallein.**—Aronson (*Centralb. med. Wiss.*, 1890, p. 577) stains sections of material hardened in liquid of Erlicki or Müller (these must be mordanted with acetate of copper) for twelve to twenty-four hours in a solution of 3 to 4 c.c. of *Gallein* (Grübler & Co.) in 100 c.c. of water with 20 of alcohol and three drops of concentrated solution of carbonate of soda. They are then differentiated by the method of Weigert, or Pal. Nerve-fibres red. A second stain with Methylen blue may follow (best after differentiating with permanganate). Similarly Schrötter (*Centralb. allg. Path.*, xiii, 1902, p. 512).

793. Schrötter (*Neurol. Centralb.*, xxi, 1902, p. 338; *Zeit. wiss. Mik.*, xix, 1903, p. 381) also stains sections for two to three hours in a 5 per cent. solution of *sulphalizarinate of soda*, to which is added a few drops of 5 per cent. oxalic acid (enough to give an orange tint), then differentiates until no more colour comes away, in carbonate of soda solution of $\frac{3}{1000}$ strength, and mounts in balsam. Myelin red, on a colourless ground.

794. **Toluidin Blue and Methylen Blue.**—Harris (*Philadelphia Med. Journ.*, May 14th, 1898) stains sections (of material hardened as for Weigert's stain) for several hours in a 1 per cent. solution of *toluidin blue* in 1 per cent. borax solution, and differentiates in saturated aqueous solution of tannic acid. Similarly, but with *methylen blue*, in a complicated way Fraenkel, *Neurol. Centralb.*, xxii, 1903, p. 766 (*Zeit. wiss., Mik.*, xx, 1904, p. 345).

Bing and Ellermann (*Arch. Anat. Phys., Phys. Abth.*, 1901, p. 260) harden in 9 parts of acetone to 1 of formol, cut without embedding, stain for five to ten minutes in saturated *Methylen blue*, and put for one or two into saturated picric acid.

795. **Other Modifications or Similar Methods.**—Flechsig, *Arch. Anat. Phys., Phys. Abth.*, 1889, p. 537; Breglia, *Zeit. wiss. Mik.*, vii, 1890, p. 36; Rossi, *ibid.*, vi, 1889, p. 182; Mercier, *ibid.*, vii, 1891, p. 480; Haug, *ibid.*, p. 153; Walsem, *ibid.*, xi, 1894, p. 236; Robertson, *ibid.*, xiv, 1897, p. 80 (*Brit. Med. Journ.*, 1897, p. 651).

Strong (*Journ. Comp. Neur.*. xiii. 1903, p. 291) finds *bichromate of copper* (of 2 to 3 per cent.) the best mordant; and that the mordanting is best done before bringing into celloidin. After staining, he treats for half a minute with osmic acid of 0·25 per cent., and differentiates as Pal.

796. Marchi's **Method (for Degenerate Nerves)** (*Rivista sperim. di Freniatria,* 1887, p. 208; *Zeit. wiss. Mik.,* ix, 1893, p. 350).—*Small pieces* of tissue are hardened for a week in solution of Müller, and then put for a few days into a mixture of 2 parts solution of Müller and 1 part 1 per cent. osmic acid solution. Sections are cut, best without imbedding, and mounted in balsam. The sheaths in normal nerves then acquire a yellow coloration, those of degenerated tracts a black one.

This process therefore gives *positive* images of the degenerated elements, Weigert's process only giving negative ones.

For a critical review of this method and its modifications see Weigert, in *Ergebnisse der Anatomie,* vii, 1897 (1898), pp. 1—8; Matuszewski, *Arch. path. Anat.,* 1905, p. 12; de Lange, *Le Nevraxe,* x, 1909, p. 83; and Lewy, *Fol. Neurobiol.,* ii, 1909, p. 471 (*Zeit. wiss. Mik.,* xxvi, 1909, p. 290).

The method has been applied to tissues that have been hardened in *formol*; but this, according to Weigert, does not seem recommendable.

Finotti (*Virchow's Arch.,* cxliii, 1896, p. 133) makes sections of material that has been in liquid of Müller for not more than a few weeks or months, and puts them for four to ten hours (in the dark) into a freshly prepared mixture of one or two parts of 1 per cent. osmic acid and one part of a concentrated solution of *picric acid* in one-third alcohol. For peripheral nerves, myelin (normal), black.

Orr (*Journ. Path. and Bact.,* vi, 1900, p. 387; *Journ. Roy. Mic. Soc.,* 1900, p. 399) treats with a mixture of 8 c.c. of 2 per cent. osmic acid, and 2 c.c. 1 per cent. *acetic acid,* which increases the penetration.

Vassale (*Arch. ital. Biol.,* 1895, p. 91) takes 75 c.c. of solution of Müller, 25 c.c. of 1 per cent. osmic acid, and 20 drops of *nitric acid.*

Nissl (*Encycl. mik. Technik,* ii, p. 248), holding that alcohol attacks the myelin, cuts without imbedding, and hurries through alcohol and bergamot oil into balsam.

Ramón y Cajal (*Trab. lab. Biol. Madrid*, ii, 1903, p. 93) has an inadmissably complicated method of treating Marchi material.

Busch (*Neurol. Centralb.*, xvii, 1898, p. 476; *Zeit. wiss. Mik.*, xv, 1899, p. 373) puts *formol material* for five to seven days into a solution of 1 part osmic acid, 3 of iodate of sodium, and 300 of water. Same stain as Marchi's, but more penetrating and sharper.

See also Venderovič, *Anat. Anz.*, xxxix, 1911, p. 414 (cuts slices of the formol material 0·5 cm. thick, and osmicates these, thus getting increased depth of reaction).

797. Osmic Acid (Exner, *Sitzb. Akad. Wiss. Wien*, 1881, lxxxiii, p. 151; Bevan Lewis, *The Human Brain*, p. 105).—A portion of brain, not exceeding a cubic centimetre in size, is placed in 1 per cent. osmic acid, and after five to ten days is cut (best without imbedding). The sections are treated by caustic ammonia (20 drops to 50 c.c. of water), which clears up the general mass of the brain substance, leaving the medullated fibres black. The preparations are not permanent. To make them so (Ranvier, *Traité*, 1 ed., p. 1086) they should be fixed for a quarter of an hour in osmic acid vapour.

798. Azoulay's **Osmic Acid Method** (*Anat. Anz.*, x, 1894, p. 25).—(a) Sections of Müller material are put for five to fifteen minutes into solution of osmic acid of 1 : 500 or 1 : 1000 strength. Rinse with water, and put them for two to five minutes into a 5 or 10 per cent. solution of tannin, warming them therein over a flame till vapours are given off, or in a stove at 50° to 55° C. Wash for five minutes in water, double-stain if desired with carmine or eosin, and mount in balsam. If the sections are too thick it will be necessary to differentiate by Pal's process, or by *eau de Javelle* diluted with 50 volumes of water. (b) Material that has been in an osmic mixture (liquid of Flemming, of Marchi, or of Golgi). Sections as before, then the tannin bath, warming for three to ten minutes, and the rest as before.

799. Heller and Gumpertz (*Zeit. wiss. Mik.*, xii, 1896, p. 385) give for peripheral nerves, and Heller (*op. cit.*, xv, 1899, p. 495) for central nervous system, the following: Sections of Müller material are put into 1 per cent. osmic acid (twenty-four hours at 37° C. for peripheral nerves; ten minutes, or thirty at the normal temperature, for central). They are treated with pyrogallic acid (a photographic developer will do) till the nerves are black, then with a violet-coloured solution of permanganate of potash till the sections become brown, then with 2 per cent. oxalic acid till they become yellow-green. Wash out well between each operation.

Similarly, TELJATNIK (*Neurol. Centralb.*, 1897, p. 521); ROBERTSON (*Brit. Med. Journ.*, 1897, p. 651; *Journ. Roy. Mic. Soc.*, 1897, p. 175), the material being previously mordanted with Weigert's chrome-alum-copper fluid for neuroglia; and ORR, *Journ. Path. and Bact.*, vi, 1900, p. 387. See also ROSSOLIMO & BUSCH, *Zeit. wiss. Mik.*, xiv, 1897, p. 55.

WITTMAACK (*Arch. Ohrenheilk.*, lxi, 1905; *Encycl. mik. Technik*, ii, p. 241) mordants till green (temporal bones) in 90 parts of Müller with 10 of *formol* and 3 to 5 of acetic acid, decalcifies with nitric acid and formol, treats sections (paraffin or celloidin) for a few minutes with osmic acid of 2 per cent., and reduces in pyrogallol of 5 per cent. Shows the least traces of myelin.

800. Iron.—ALLERHAND (*Neurol. Centralb.*, xvi, 1897, p. 727; *Encycl. mik. Technik.*, p. 944) puts sections of Müller material for fifteen minutes into warm 50 per cent. solution of *Liquor ferri sesquichlorati*. then for an hour or two into 20 per cent. tannin solution (old and brown). They are then differentiated by the method of PAL, taking, however, the liquids twice as strong.

An iron-alum process is described by STRONG in *Journ. comp. Neurol.*, xiii, 1903, p. 291.

801. Silver Nitrate.—VASTARINI-CRESI (*Att. Accad. Med.-Chir. Napoli*, 1, 1896) hardens in formol, cuts thick sections, washes them with 40 per cent. alcohol, puts them in the dark into 1 per cent. solution of nitrate of silver in alcohol of 40 to 70 per cent., then washes thoroughly.

Similarly, MOSSE (*Arch. mik. Anat.*, lix, 1901, p. 401), impregnating bichromic material with 1 per cent. solution of *argentamin*, and reducing in 10 per cent. pyrogallic acid, and differentiating by the method of PAL. (Argentamin is an alkaline solution of equal parts of phosphate of silver and ethylendiamin in 10 parts of water).

802. Polarisation.—Myelin can sometimes be detected in *fresh material* by the polariscope, see AMBRONN & HELD, *Ber. Math. Phys. Ges. Wiss. Leipzig.*, 1895, p. 37, and GAD & HEYMANS, *Arch. Anat. Phys., Phys. Abth.*, 1890, p. 531.

Myelin-and-axis-cylinder Stains.

803. Methylen Blue.—Sahli (*Zeit. wiss. Mik.*, 1885, p. 1) stains sections of tissue hardened in bichromate for several hours in concentrated aqueous solution of methylen blue, rinses with water, and stains for five minutes in saturated aqueous solution of Säurefuchsin. If now the sections be rinsed with alcohol and brought into a liberal quantity of water, the stain becomes differentiated, axis-cylinders being shown coloured red and the myelin sheaths blue.

Or, the sections are stained for a few minutes or hours in:

Water	40 parts.
Saturated aqueous solution of methylen blue	24 ,,
5 per cent. solution of borax . . .	16 ,,

—then washed either in water or alcohol until the grey matter comes out, cleared with cedar oil and mounted in balsam.

804. Säurefuchsin.—Finotti (*Virchow's Archiv*, cxliii, 1896, p. 133) stains strongly in Delafield's hæmatoxylin, then for a few seconds in concentrated solution of picric acid, then in 0·5 per cent. Säurefuchsin, and treats with alkaline alcohol (caustic potash).

Ohlmacher (*Journ. Exper. Med.*, ii, 1897, p. 675) stains sections for one minute with anilin-water gentian, then for a few seconds in a solution of 0·5 per cent Säurefuchsin in saturated solution of picric acid diluted with one volume of water, and differentiates with alcohol and clove oil.

Kaplan (*Arch. Psychiatr.*, xxxv, 1902, p. 825) mordants (for months) in Müller, stains sections for a day or more in ⅓ per cent. aqueous Säurefuchsin, rinses in water acidulated with HCl, and differentiates by the method of Pal (permanganate and potassium sulphite).

805. Safranin.—Adamkiewics (*Sitzb. k. Akad. Wiss. Wien. Math. Naturw. Kl.*, 1884, p. 245 ; *Zeit. wiss. Mik.*, 1884, p. 587).—Stains sections of Müller material in concentrated solution of *safranin*, differentiates in alcohol and clove oil, brings back again into water, washes in water acidified with acetic acid, and stains in *methylen blue.* Myelin red, nuclei violet.

Similarly Ciaglinski (*Zeit. wiss. Mik.*, viii, 1891, p. 19) and Steoebe (*ibid.*, x, 1893, p. 336), the former employing safranin followed by anilin blue, whilst the latter first stains with anilin blue, then differentiates with alcohol containing a very little caustic potash, and after-stains with safranin.

806. Congo Red.—Nissl (*Zeit. wiss. Mik.*, 1886, p. 398) stains for 3 days in Congo red (5 parts to 400 of water) and differentiates in alcohol with 3 per cent. of nitric acid.

807. Other Methods.—For Paladino's *palladium chloride* methods see *last ed.*, or *Rendic R. Accad. Scienze*, Napoli, iv, 1890, p. 14, and 1891 [1892], p. 227 ; and *Boll. Accad. Med. Roma*, xix, 1893, p. 256 ; *Arch. Ital. Biol.*, xx, 1894, p. 40.

For Wolters' *vanadium chloride* process see next chapter.

CHAPTER XXXIV.

AXIS-CYLINDER AND DENDRITE STAINS (GOLGI AND OTHERS).

808. Introduction.—There are three chief methods for the *anatomical* (§ 736) study of axis-cylinders and protoplasmic nerve-cell processes, viz. the methylen-blue *intra-vitam* method, the bichromate-and-silver method of GOLGI, and the bichromate-and-sublimate method of GOLGI. The two latter, with some other methods suitable for the same or similar purposes, form the subject of this chapter.

809. The Methods of GOLGI. There are two methods of GOLGI, viz. the **Bichromate and Nitrate of Silver Method** and the **Corrosive Sublimate Method.**

The bichromate and nitrate of silver method has been worked out by GOLGI in *three* forms—the *slow* process, the *rapid* process, and the *mixed* process.*

The rapid process is the one that is the most in use at the present time, and may be taken to be the classical method of inquiry into the finer relations of the neurons in *hardened* tissue.

General characters of the impregnation.—The preparations have not in the least the appearance of stains, and are even very different in aspect from the impregnations obtained by the ordinary methods of impregnating with silver or gold.

---

* In a recent text-book, the *Leitfaden* of RAWITZ, the sublimate method is called "the slow method of GOLGI," and the bichromate and silver nitrate method is given under the form of the slow process, and called "the rapid method of GOLGI." RAWITZ further attributes the rapid method to RAMÓN Y CAJAL, which is equally erroneous. Similar confusions are made by MERCIER in his *Coupes du Système Nerveux Central*, and by POLLACK and other authors. Valuable accounts of the silver method have been given by v. LENHOSSÉK in his *Feinere Bau d. Nervensystems*, 2nd edit., 1895, and by KALLIUS in the art. "Golgische Methode" in the *Encycl. mik. Technik.*, 1903.

The impregnation is a *partial* one, by which is meant that of all the elements, whether nervous or not, that are present in a preparation, only a limited number are coloured. That is one of the great advantages of the method. For if all the elements present were coloured equally, you would not be able to see the wood for the trees, for you would hardly be able to follow any one element for more than a very short distance. But Golgi's method selects from among the elements present a small number which it stains with a great intensity and very completely—that is to say, throughout a great length, so that they are both very clearly separated from those elements that have remained uncoloured, and also can be followed out for a great distance.

Axis-cylinders are *generally* impregnated only so long as they are *not medullated*. In the adult the method stains nerve-cells and their processes, so far as these are not myelinated; but if it be wished to impregnate the axis-cylinders of the cerebro-spinal axis the method is best applied to embryos or new-born animals at a time when the fibres have not become surrounded by their sheath of myelin.

There is no other method which will allow cell-processes to be followed out for such great distances. But the method does not demonstrate at the same time the histological detail of other tissues that may be present in the preparations, and all cytological detail is lost. It is *par excellence* a *special* method.

Nervous tissue is not the only thing that is impregnated in these preparations; neuroglia, connective tissue, fibrils, etc., are impregnated, and the method has been applied with success to the study of such things as bile-capillaries, gland-ducts, and the like. Both on account of this character, and on account of the capriciousness with which the impregnation takes hold of only certain elements of the preparations, care must be exercised in the interpretation of the images obtained. A further source of possible error is found in the fact that the method frequently gives precipitation-forms of the silver salt that simulate dendrites and other structures (see FRIEDLAENDER in *Zeit. wiss. Mik.*, xii, 1895, p. 168, and the plate in the following number.)

The method has been applied with success to the tissues of Invertebrates—Insects, *Lumbricus, Tubifex, Helix, Limax, Distomum, Astacus, Actinida, etc.*

The method has been described at length by Golgi in the *Archives Italiennes de Biologie,* t. iv, 1883, p. 32 *et seq.,* and vii, 1886, p. 15 *et seq.* The following account is from the latter paper. The earlier form of the method should not be followed.

810. Golgi's **Bichromate and Nitrate of Silver Method,** *SLOW Process* (*loc. cit.,* p. 17).—(*a*) *The hardening.*—The tissues must be hardened in a bichromate solution. Either pure bichromate of potash may be employed, or liquid of Müller (the reaction can be obtained with liquid of Erlicki, but it is not to be recommended). The normal practice is to take bichromate of potash, beginning with a strength of 2 per cent., and changing this frequently for fresh solutions of gradually increased strength, 2½, 3, 4, and 5 per cent. The tissue should be *as fresh as possible;* though satisfactory results may sometimes be obtained from material taken twenty-four to forty-eight hours after death.* *It should be in pieces of not more than 1 c.cm. or 1½ c.cm. in size.*

The most difficult point of the method consists in *hitting off the exact degree of hardening* in the bichromate that should be allowed before passing to the next stage. In summer good results may be obtained after fifteen to twenty days, and the material may continue in a favourable state for impregnation up to thirty, forty, or fifty days. In cold weather good results can seldom be obtained under a month: when obtained, the material may continue to give good results up to two, three, and even four months of hardening. The only way to make sure is to pass trial portions of the tissue at intervals into the silver-bath, in summer frequently, in winter every eight or ten days, and observe whether the reaction is obtained.

Good results are obtained by *injecting* the organs with the hardening fluid (2·5 per cent. bichromate). See § 737.

Stoving at a temperature of 20° to 25° C. is useful for abridging the hardening, but there is risk of over-hardening; and GOLGI thinks the results are never quite so delicate as after hardening in the cold.

---

* Material that has been hardened in formol may also be used. See § 817 (Gerota and Bolton), and v. Lenhossék's *Feinere Bau d. Nervensystems,* p. 23. I have had good results with material that had been three months in formol (I have not tried older).

(*b*) *Impregnation.*—As soon as the pieces of tissue have attained the proper degree of hardening, they are brought into a bath of nitrate of silver. The usual strength of this bath is 0·75 per cent., but 0·50 per cent. may be taken for material that has not been quite enough hardened, and solutions of 1 per cent. may be used for material that has been slightly over-hardened. The solution *may* be acidified (see RAMÓN Y CAJAL, § 819).

A *large quantity* of solution should be taken for the bath.

The moment the pieces of tissue are put into the silver-bath an abundant precipitate is formed. This of course weakens the bath *pro tanto.* It is therefore well, before putting the pieces into the final silver-bath, to first wash them well in a weaker silver solution, until on being put into a fresh quantity of it no further precipitate is formed. Used solutions will do for this purpose. The final silver-bath in general needs no further attention, unless it be that sometimes, in the case of tissues that have taken up a great deal of bichromate of potash, the solution may after six to ten hours become somewhat yellow, in which case it should be changed for fresh.

It is not necessary to keep the preparations in the dark during the impregnation bath; in winter it is well to keep them in a warm place.

The time normally *necessary* for impregnation by the silver is from twenty-four to forty-eight hours (forty-eight being quite exceptional). But tissues may remain in the bath without hurt for days, weeks, or months.

(*c*) *Preservation.*—As soon as a trial has shown that a sufficiently satisfactory impregnation has been obtained the pieces are brought into alcohol. The alcohol is changed two or three times, or even more, until it remains transparent even after the preparations have been two or three days in it; for in view of good preservation it is necessary that the excess of nitrate of silver should be washed out from them thoroughly.

Sections are now made (see § 821). They are to be washed very thoroughly in three or four changes of absolute alcohol. They are then cleared, first in creasote, in which they should remain only a few minutes, then in oil of turpentine, in which they should remain for ten to fifteen minutes (they may remain there for days without hurt). They are then mounted in damar (rather than in balsam), *and without a cover.* Preparations mounted under covers in the usual way always go bad sooner or later, whilst those that are mounted without a cover keep very well, *especially if they be kept in the dark.* GOLGI states that he has a large number that have kept without change for nine years.

As a general rule *thick* sections (50 to 60 μ or more) show much more than thin ones, but *do not seem to keep so well.*

The order in which the elements of tissues impregnate is generally—first, axis-cylinders, then ganglion cells, and lastly neuroglia cells.

811. GOLGI'S **Bichromate and Nitrate of Silver Method,** *RAPID process* (*op. cit.*, p. 33). *Small pieces* of very fresh tissue are thrown into—

Bichromate solution of 2 to 2·5 per cent. strength . . . . . 8 parts.
Osmic acid of 1 per cent. strength . 2 „

(Or, later, two parts of bichromate of 3 per cent. to 1 of the osmic acid.) The tissues begin to be in a fit state for taking the silver impregnation from the second or third day; in the next following days they are in a still more favourable state, but the favourable moment does not last long; the faculty of impregnation soon declines, and is generally quite lost by the tenth or twelfth day.

The silver impregnation is conducted exactly as in the slow process, and sections are prepared and mounted in the same manner. (I find that they should not be left in alcohol for more than an hour or so before mounting.)

There is this difference, that the *impregnated material* cannot be preserved for any length of time in alcohol, but *must not remain for more than two days in it.* But it may be kept in the *silver solution* until wanted for sectioning. According to VAN GEHUCHTEN (*La Cellule*, vi, 1890, p. 405), material may be kept for six months in the silver, *with advantage,* showing abundant reductions where none were found after forty-eight hours. But it *must be kept in the dark.*

The following notes as to the proper duration of the hardening process in different cases are taken from the papers quoted and other sources, most of which may be found in v. LENHOSSÉK, *op. cit.*, p. 23.

Spinal cord of chick, from the sixth to the tenth day of incubation—twelve to forty-eight hours in the mixture (up to the fifth day the embryos may be treated whole, later the vertebral column should be dissected out and cut into two or three segments; it need not be opened). The spinal column of new-born rats and mice should be treated in the same way, and remain in the mixture for twenty-four hours (for spinal ganglia), or for two to six days for the cord itself. (The encephalon of these subjects may be treated in just the same way, without being dissected out.)

VON LENHOSSÉK (*op. cit.*, p. 10) recommends for human (fœtal) cord two to three days for neuroglia, three to five for nerve-cells, and five to seven for nerve-fibres and collaterals.

Cerebellum of new-born subjects, three to five days in the mixture.

Cerebral cortex of young subjects, two to three days (Mice), or as much as five (Rabbit, Cat); cortex of adults, eight to fifteen days. The most favourable region of the brain is the *cornu Ammonis*, especially in the Rabbit.

Retina—twenty-four to forty-eight hours in the mixture, then "double" impregnation (§ 815).

Sympathetic—three to seven days in the mixture, and two in the silver: then double impregnation.

Spinal cord of larvæ of Amphibia. The entire larvæ (best 2 to 2·5 centimetres long) should be put for two to five days into the mixture, and for one to two into the silver.

Epidermis of *Lumbricus*—three to six days in the mixture, and two in the silver, or double impregnation if necessary. SMIRNOW makes the mixture of equal parts of 5 per cent. bichromate and 1 per cent. osmic acid, and leaves in it for five to twenty-eight days, and one to two days in the silver (0·75 per cent.).

Nervous system of *Helix* (glia-cells). The above mixture for eight to ten days, then silver of 0·75 to 1 per cent.

As a general rule, the younger the subject, the shorter should the hardening be. If it has been too short, sections will have a brownish-red opaque aspect, with precipitates, and irregular impregnation of cells and fibres. If it has been too long, the ground will be yellow, without precipitates, but with no impregnated elements, or hardly any.

This process has the advantage of great rapidity, and of sureness and delicacy of result, and is the one that has found the most favour with other workers. But for methodical study of any given part of the nervous system GOLGI himself prefers the following :

812. GOLGI'S **Bichromate and Nitrate of Silver Method,** *MIXED Process* (*op. cit.*, p. 34).—Fresh pieces of tissue are put for periods varying from two to twenty-five or thirty days into the usual bichromate solution (§ 810). Every two or three or four days some of them are passed on into the osmio-bichromate mixture of the rapid process, hardened therein for from three or four to eight or ten days, and finally impregnated with silver, and subsequently treated exactly as in the rapid process.

The reasons for which GOLGI prefers this process are—the certainty of obtaining samples of the reaction in many stages of intensity, if a sufficient number of pieces of tissue have been operated on ; the advantage of having at one's disposition a notable time—some twenty-five days—during which the tissues are in a fit state for taking the silver, and the possibility of greatly hastening the process whenever desired by simply bringing the pieces over at once into the osmic mixture ; lastly, a still greater delicacy of result, especially remarkable in the demonstration of axones.

813. **Theory of the Impregnation.**—It used to be held that the reaction depends on the formation in the tissues of a *precipitate* of some salt of silver. But this seems incorrect. I find (in accordance with LENHOSSÉK, 'Feinere Bau d. Nervensystems,' p. 19) that the coloration is not due to a visible precipitate, but is a true stain, accompanied (in unsuccessful impregnations) by a precipitate which does not help the stain but is injurious to it. It has been maintained that the stain is merely superficial, and the method has been called an "incrustation method." I find that it extends throughout the whole thickness of the impregnated elements.

The chemical nature of the stain has not been made out.

A critical review of the Golgi method by WEIGERT may be found in *Ergebnisse der Anatomie*, v, 1895 (1896), p. 7.

See also HILL (*Brain*, part 73, 1896, p. 1), AZOULAY (*Comptes Rend.*

Soc. Biol. [10], i, 1894, p. 839); and KALLIUS (*Encycl. mik. Technik.*, *Art.* "Golgi'sche Methode."

Modifications concerning the Impregnation of the Tissues.

814. RAMÓN Y CAJAL (*Zeit. wiss. Mik.*, vii, 1890, p. 332) gives 3 per cent. as the strength of the bichromate in the mixture for the *rapid* process, and in numerous other places has given it as 3·5 per cent. This latter strength has been adopted by most of the workers who use the rapid process, and the mixture containing this proportion of bichromate is generally known as RAMÓN Y CAJAL'S mixture.

815. RAMÓN Y CAJAL'S **Double-Impregnation Process** (*Trab. Lab. Hist. Med. Barcelona*, 1891; *La Cellule*, vii, 1891, p. 130).—Sometimes the usual rapid method fails to give good impregnations. These, however, may frequently be obtained by putting the tissues back for a day or two into the osmium-bichromate mixture, or into a weaker one containing only two parts of osmic acid solution to 20 of the bichromate. After this they are washed quickly with distilled water, and put for a second time into the silver solution for thirty-six to forty-eight hours. It is important to hit off the proper duration of the first impregnation in the bichromate. If it has been too long (four days) or too short (one day), the second impregnation will not succeed. In this case a third impregnation must be resorted to, the objects being again treated with the weak osmium-bichromate mixture, and afterwards again with the silver solution.

This modification of the original process is the most important that has hitherto been made.

816. GOLGI'S **Process for Rejuvenation of Over-hardened Tissues.**—Tissues that have been much too long in the osmium-bichromate mixture will no longer take on the silver impregnation. They can, however, be rejuvenated and made to impregnate in the following manner, due to GOLGI, and published by SACERDOTTI (*Intern. Monatsschr.*, xi, 1894, p. 326). They are washed in a half-saturated solution of acetate of copper until they no longer give a precipitate, and are then put back again for five or six days into the osmium-bichromate mixture. Sections, it is said, will bear mounting in thickened oil of cedar under a cover.

More recently (*Cinquantenaire Soc. Biol.*, 1899, p. 514) Golgi puts for some hours or days into a mixture of equal parts of bichromate of 2 to 3 per cent. and sulphate of copper of 4 to 5 per cent., or into the cupric mixture used for the intra-cellular network, § 777.

Gemelli (*Anat. Anz.*, xliii, 1913, p. 414) takes a mixture of "acetate of copper (4 per cent.) and bichromate of potash (5 per cent.)." Time not stated.

817. Formaldehyde for the Rapid Process.—Strong (*Anat. Anz.*, x, 1895, p. 494) finds that formaldehyde can with advantage be *substituted for the osmic acid* in the osmio-bichromic mixture of Golgi's rapid process. He adds from 2·5 to 5 per cent. of formalin to the (3·5 to 5 per cent.) bichromate solution.

The advantage is that the stage of hardening favourable for impregnation lasts longer; in other words, the formaldehyde bichromate does not over-harden.

Durig (*ibid.*, p. 659) obtained the best results by means of 3 per cent. bichromate solutions containing 4 to 6 per cent. of formaldehyde, hardening therein for three days, and after silvering for two days putting back into the mixture and proceeding as in Ramón y Cajal's double impregnation process.

Fish (*Proc. Amer. Mic. Soc.*, xvii, 1895, p. 319) takes:

Formalin	2 c.c.
3 per cent. bichromate . . .	100 "

leaving the tissues three days in this liquid and three days in the silver nitrate (¾ per cent.).

Or, with advantage:

Liquid of Müller	100 c.c.
10 per cent. formalin . . .	2 "
1 per cent. osmic acid . . .	1 "

Odier (*La Rachicocainisation*, Genève, 1903, p. 27) takes two parts of undiluted formalin, instead of the 10 per cent.

The formalin and bichromate mixtures should be kept in the dark. It is well only to make them up *at the instant of using* them. Odier finds these mixtures afford a more abundant impregnation, with fewer precipitates.

Kopsch (*Anat. Anz.*, xi, 1896, p. 727) takes 4 parts of 3·5 per cent. bichromate solution, and 1 of commercial formaldehyde solution, and after twenty-four hours transfers to pure 3·5 per cent. bichromate for at least 2 days (retina), or 3 to 6 (central organs). He finds that by this means precipitates are almost entirely avoided. This I also find, but I seem to get a too abundant impregnation of capillaries.

Gerota (*Intern. Monatsschr. Anat.*, xiii, 1896, p. 108) *first* hardens (brain) for a week or two in 5 to 10 per cent. formol solution, *then* puts small pieces for three to five days into 4 per cent. bichromate, then into the silver.

Similarly Bolton (*Lancet*, 1898, p. 218; *Journ. Roy. Mic. Soc.*, 1898, p. 244).

Schreiber (*Anat. Anz.*, xiv, 1898, p. 275) obtained good results (on appendages of Crustacea which were impervious to the osmic mixture) with mixtures of five parts 2·5 per cent. bichromate to one of 4 per cent. formaldehyde, or one part 2·5 per cent. bichromate to two of 5 per cent. formaldehyde, the specimens remaining for one day in the first, for two days in the second.

Similarly Duboscq (*Arch. z. Exper.*, 1899, p. 483), warming the mixture to 40° C.

van Gehuchten (*in litt.*), and other observers, *have not obtained good results* with formaldehyde.

Kallius (*Encycl.*, p. 564) finds these mixtures good for brain, but not so much so for other organs.

818. Acetic Aldehyde.—Vassale and Donaggio (*Monitore Zool., Ital.*, vi, 1895, p. 82) harden pieces of at most 1 cm. in thickness for fifteen to twenty days in a mixture of five parts of aldehyde with 100 of 3 to 4 per cent. bichromate, changing the fluid after a few days, as soon as it has become dark. The rest as Golgi.

819. Modifications of the Silver Impregation.—Ramón y Cajal (*Rev. trim. Hist.*, No. 2, 1888, note) found the addition of a very little formic acid to the silver bath facilitated reduction. According to van Gehuchten (*La Cellule*, vii, 1891, p. 83), 1 drop of the acid should be added to 100 c.c. of the silver. But the practice is now generally abandoned.

Berkeley (*Johns Hopkins Hosp. Rep.*, vi, 1897, p. 1; *Journ. Roy. Mic. Soc.*, 1898, p. 242) impregnates, after hardening in the osmio-bichromate, in a freshly prepared solution of two drops of 10 per cent. phosphomolybic acid to 60 c.c. of 1 per cent. silver nitrate, which in winter should be kept at a temperature of about 26° C.

Hill (*op. cit.*, § 813) takes instead of silver nitrate a $\frac{3}{4}$ per cent. solution of silver *nitrite*, with 0·1 per cent. of formic acid added.

Gudden (*Neurol. Centralb.*, xx, 1901, p. 152) takes the lactate of silver (sold as "actol.") and finds it much more penetrating.

Fajerstajn (*ibid.*, p. 98) uses ammonio-nitrate in a complicated way.

820. Avoidance of Precipitates.—Golgi's process frequently gives rise to the formation at the surface of the preparations of voluminous precipitates that are destructive of the clearness of the images. Sehrwald (*Zeit. wiss. Mik.*, vi, 1889, p. 456) has found that this can be avoided as follows. A 10 per cent. solution of gelatin in water is made. The tissues are coated with this, by dipping and cooling several times, or are imbedded in it, in a paper imbedding box, with the aid of a little heat, and are brought therein into the silver-bath. After the silvering the gelatin is removed before cutting by warm water saturated with chromate of silver.

Martinotti wraps the tissue simply in blotting-paper, but this does not appear to be efficacious.

Athias takes wafer-papers.

Ramón y Cajal covers tissues with a layer of congealed blood, which need not be removed before cutting, or with collodion, or peritoneal membrane. See *Retina*.

Modifications concerning the Preservation of the Preparations.

821. Cutting.—The chief quality of Golgi's process is that it admits of the following of nerve-cell processes for a *very great distance.* Evidently this cannot be done with very *thin* sections. And as sufficiently thin ones can be obtained without imbedding, the general practice is simply to wash the preparations taken from the silver-bath with water, fix them to a cork with gum, put the whole into alcohol for a few minutes to harden the gum, and cut with a microtome without imbedding.

But imbedding is possible, if it can be got through rapidly enough. Pieces of tissue as small as possible should be dehydrated in from half an hour to two hours, put for the same time into thin celloidin, then coated with thick celloidin, gummed on a cork and cut, the sections being collodionised if necessary. Thin specimens such as retina may be soaked for a short time in celloidin, put between two slabs of solid celloidin lightly pressed together, and the whole cut after a short treatment with alcohol of 70 per cent. Similarly with paraffin. The tissues should be got quickly through the lower grades of alcohol, and not remain for more than a few hours in alcohol of 95 per cent. or absolute. They should be cleared with cedar oil (xylol attacks the impregnation), and put direct into paraffin of as low a melting point as possible. The cedar oil should be used over and over again, as it takes up a little silver (see BROOKOVER, *Journ. Comp. Neurol.*, xx, 1910, p. 49).

822. **Mounting.**—Without special precautions, the stain will not keep in sections mounted *under a cover* in the usual way. An elaborate discussion (for which see *previous editions*) between SEHRWALD (*Zeit. wiss. Mik.*, vi, 1890, p. 443), SAMASSA (*ibid.*, vii, 1890, p. 26), and FICK (*ibid.*, viii, 1891, p. 168) furnishes the net practical result that *watery fluids* should be avoided as much as possible during the after-treatment, and that sections should either be *mounted without a cover*, or *on* a cover raised free of contact with the slide by means of wax feet or the like, or, for study, inverted over the aperture of a hollowed-out wooden slide; or that the balsam of the mount should be *rendered perfectly anhydrous* by careful heating on the slide, with the section in it, until it immediately sets hard on cooling, before the cover is applied.

This last method is also recommended by HUBER (*Anat. Anz.*, vii, 1892, p. 587). I think it is safer to keep the mount uncovered till the sections have become quite dry in it, and the balsam (applied from time to time in thin layers) quite hard; then cover with a warmed cover pressed down.

But if mounting under a cover at once be preferred, one of the following methods may be employed.

823. **GREPPIN'S Process** (*Arch. Anat. Entw. Anat. Abth.*, 1889, Supp., p. 55).—Sections are treated for thirty to forty seconds (until whitish) with 10 per cent. solution of hydrobromic acid, and then well washed in several changes of water and mounted under a cover in the usual way. They can be further reduced in sunlight if desired. Further details in *previous editions*.

824. **OBREGIA'S Process** (*Virchow's Archiv*, cxxii, 1890, p. 387).—Sections are brought from absolute alcohol into a mixture of eight to ten drops of 1 per cent. solution of gold chloride with 10 c.c. of absolute alcohol, which should be prepared half an hour beforehand and exposed to diffused light until the sections are placed in it, when it should be

put into the dark. After fifteen to thirty minutes therein, according to their thickness, the sections are quickly washed in 50 per cent. alcohol, then in water, then treated for five or ten minutes (not more) with 10 per cent. solution of hyposulphite of soda. They are lastly washed well with water, and may be then mounted at once in balsam under a cover, or if desired may be previously stained with carmine or hæmatoxylin, or Pal's modification of Weigert's process, or the like.

825. Kallius (*Anat. Hefte*, ii, 1892, p. 271) has worked out the following process. Take 20 c.c. commercial hydroquinone developing solution and 230 c.c. distilled water (the hydroquinone solution may be made up with 5 grms. hydroquinone, 40 grms. sodium sulphite, 75 grms. carbonate of potassium, and 250 grms. distilled water). At the instant of using, further dilute the solution with one third to one half its volume of absolute alcohol, and put the sections (from which the unreduced silver has been removed as far as possible by washing in many changes of alcohol) into it for several minutes; they become dark grey to black. They are then put for ten to fifteen minutes into 70 per cent. alcohol, then brought for five minutes into solution of hyposulphite of soda (about 10 parts to 50 of water), and thence into a large quantity of distilled water, where they should remain for twenty-four hours or more. Lastly, dehydrate in the usual way and mount under a cover. After-staining with carmine, etc., may be employed.

Curreri (*Anat. Anz.*, xxxii, 1908, p. 432) after fixing tones for a short time in 0·7 grms. gold chloride, 3 grms. sodium acetate, and 100 c.c. water.

826. Zimmermann's **Process** (*Arch. mik. Anat.*, lii, 1898, p. 554).—Sections are brought from alcohol into a *large quantity* of a mixture of 1 part physiological salt solution and 2 parts 96 per cent. alcohol. They must be kept in motion therein for ten to fifteen minutes, after which they are brought into alcohol of 75 to 96 per cent. in a bright light, until they have become dark (half a day). They may be after-stained with thionin (cells blue).

Later (*Arch. Mik. Anat.*, lxxviii, 1911, p. 199) he reduces in 20 c.c. of saturated solution of carbonate of soda with 0·5 grm. of adurol for several hours, and after-stains with hæmalum or alum cochineal.

The Sublimate Method.

827. Golgi's **Bichromate and Sublimate Method** (*Archivio per le Scienze Mediche*, 1878, p. 3; *Archives Italiennes de Biologie*, iv, 1883, p. 32; vii, 1886, p. 35).—For hardening, use either a solution of bichromate of potash progressively raised from 1 per cent. to 2½ per cent., or Müller's solution. It is *best* to take small pieces of tissue (not more than 1 to 2 c.c.), large quantities of liquid, and change the latter frequently. But the reaction can be obtained with much larger pieces,

even entire hemispheres. In this case the brain should at first be treated by repeated *injections* of the liquid. Fifteen to twenty days' immersion will suffice, or even six to eight, but twenty to thirty should be preferred, and an immersion of several months is not injurious.

The tissues when hardened are passed direct from the bichromate into 0·5 per cent. solution of bichloride of mercury. An immersion of eight to ten days therein is necessary in order to obtain a complete reaction (or for entire hemispheres two months or more). The solution must at first be changed every day, and later on as often as it becomes yellow. At the end of the reaction the preparations will be found decolourised, and offering the aspect of fresh tissue. They may be left in the bichloride for any time.

In *Rendiconti R. Ist. Lombardo di Sci. Milano*, 2, xxiv, 1891, pp. 594, 656 (see *Zeit. wiss. Mik.*, viii, 3, 1891, p. 388), GOLGI says that for the study of the "diffuse nervous reticulum" of the central nervous system the best results are obtained by keeping the preparations in 1 per cent. sublimate for a very long time, two years being not too much in some cases.

The reaction may be said to have begun by the time the tissues are nearly decolourised. From that time onwards sections may be made day by day and mounted if successful.

Before mounting, the sections must be repeatedly washed with water, otherwise they will be spoilt by the formation of a black precipitate. (In the last place quoted GOLGI says that after washing they may be toned by putting them for a few minutes into a photographic fixing-and-toning bath, after which it is well to wash them again, and stain them with some acid carmine solution.) Mount in balsam or (preferably) glycerin.

The elements acted on are—(1) The ganglion cells, with all their processes and ramifications. (2) Nuclei, which is not the case with the silver process. (3) Neuroglia cells. But the reaction in this case is far less precise and complete than that obtained by the silver process. (4) The blood-vessels, and particularly their muscular fibre cells.

The method is said to give *good* results only with the cortex of the cerebral convolutions, hardly any results at all with the spinal cord, and very scanty results with the cerebellum. It is superior to the silver method in that the reaction can always be obtained with perfect *certainty* in a certain time; that the preparations can be perfectly preserved by the usual methods; and that *large* pieces of tissue can be impregnated. It generally gives a more *abundant* impregnation than the silver method.

See also Flatau, in *Arch. mik. Anat.*, xlv, 1895, p. 158.

Modifications of Golgi's Bichromate and Sublimate Method.

828. Pal [*Erratim* "Tal," *loc. cit.*] (*Gazz. degli Ospitali*, 1886, No. 68) finds that if sections made by this process be treated with solution of sodium sulphide, a much darker stain is obtained. Sections may then advantageously be double-stained with Magdala red.

Golgi's method may be combined with Weigert's nerve stain (see Pal, *Wien. med. Jahrb.*, 1886; *Zeit. wiss. Mik.*, v, 1887, p. 93).

For Flechsig's modifications, see *Arch. Anat. Phys., Physiol. Abth.*, 1889, p. 537.

829. Cox (*Arch. mik. Anat.*, xxxvii, 1891, p. 16) finds the sublimate and bichromate may be used *together*. He used a fluid consisting of 20 parts 5 per cent. bichromate, 20 parts 5 per cent. sublimate, 16 parts 5 per cent. simple chromate of potash, and 30 to 40 parts of water. (The chromate should be diluted with the water before adding it.) The mixture should be as little acid as possible. The pieces of tissue should be *small*. The duration of the impregnation is from two to three months. There is considerable difficulty in preserving sections, which are best made with a freezing microtome, alcohol being avoided, treated for an hour or two with 5 per cent. solution of sodium carbonate, and mounted *without a cover*, in a medium composed of—gum sandarac, 75 gr.; camphor, 15; oil of turpentine, 30; oil of lavender, 22·5; alcohol, 75; castor oil, 5–10 drops. For examination, add a drop of castor oil, and cover.

Dr. A. Sanders writes me (June, 1898) that the stain keeps very well if the sublimate be well removed by washing in many changes of alcohol, and the tissues passed through alcohol and ether into celloidin, and the sections mounted in chloroform-balsam under a cover. I find the *stain* keeps; but the preparations quickly develop opaque granules that are very undesirable.

Bremer (*Anat. Rec.*, 1910, p. 265) cuts in celloidin and stains with alum hæmatein and eosin.

For the very complicated platinum-substitution processes of Robertson and Macdonald see *Journ. Ment. Sci.*, xlvii, 1901, p. 327; or *Journ. Roy. Mic. Soc.*, 1902, p. 501.

830. Ziehen's **Gold and Sublimate Method** (*Neurol. Centralb.*, x, 1891, p. 65).—Small pieces of fresh material are thrown into a large quantity of a mixture of 1 per cent. sublimate solution and 1 per cent. chloride of gold solution in equal parts. They remain therein for at least three weeks, preferably for several months (up to five), by which time they will have become of a metallic red-brown colour. They are gummed on cork and sectioned without imbedding. The sections are treated either with Lugol's solution diluted with four volumes of water, or with dilute tincture of iodine, until duly differentiated, then washed and mounted in balsam. Both medullated and non-medullated nerve-fibres are stained, also nerve and glia cells and their processes.

831. Krohnthal's **Lead Sulphide Impregnation** (*Neurol. Centralb.*, xviii, 1899, No. 5; *Zeit. wiss. Mik.*, xvi, 1899, p. 235) consists in treating tissues first with formate of lead and then with hydric sulphide. The formate is prepared by dropping formic acid slowly into solution of acetate of lead. White crystals of formate of lead are abundantly formed; the mother liquor is filtered off, and the crystals are dissolved to saturation in water. The solution is mixed with an equal volume of 10 per cent. formol; pieces of brain or spinal cord are put into the mixture for five days, and are then brought direct into a mixture of equal parts of 10 per cent. formol and hydric sulphide solution. After five days therein they are cut in celloidin, and the sections mounted in xylol-balsam under a cover. They seem to be quite permanent. Nerve-cells as well as nerve-fibres are impregnated. The impregnation is a very complete one.

Corning (*Anat. Anz.*, xvii, 1900, p. 108) hardens the tissues with 10 per cent. formol before bringing them into the formol-formate mixture, and so obtains better results. He obtains his formate of lead direct from Merck (*Plumbum formicicum*). He prefers to cut without imbedding. Other details *loc. cit.*

832. **Wolter's Chloride of Vanadium** process (*Zeit. wiss. Mik.*, vii, 1891, p. 471) :

The material (either central or peripheral nervous tissue) is *hardened in the bichromate liquid of* Kultschitzy, § 55, followed by alcohol, *as there described.* Sections are mordanted for twenty-four hours in a mixture of 2 parts of 10 per cent. solution of chloride of vanadium and 3 parts of 3 per cent. solution of acetate of aluminium, washed for ten minutes in water, and stained for twenty-four hours in a solution of 2 grms. of hæmatoxylin (dissolved in a little alcohol) in 100 c.c. of 2 per cent. acetic acid. They are washed out until they are of a light blue-red colour in 80 per cent. alcohol acidulated with 0·5 per cent. of hydrochloric acid. Remove the acid thoroughly by washing with pure alcohol, dehydrate, clear with origanum oil, and mount.

Chiefly an axis-cylinder stain, myelin being coloured only if the differentiation in the acid alcohol is insufficient, but cells are also stained.

833. **Methylen Blue.**—Meyer (*Arch. mik. Anat.*, xlvi, 1895, p. 282, and xlvii, 1896, p. 734) has obtained good results (for the *central* nervous system, not for the peripheral) by means of *subcutaneous* injection. *Large* quantities of solution must be injected, in several portions, at intervals of one to several hours. After some time the organs should be thrown direct into the bath of Bethe, § 344, and remain in it till the next day.

Ramón y Cajal (*Rev. Trim. Micr.*, Madrid, i, 1896, p. 123; *Zeit. wiss. Mik.*, xiv, 1897, p. 92) stains by "propagation" or "diffusion." The brain is exposed (rabbit) and the cortex is divided into slices of a couple of millimetres thickness by means of a razor. The slices are then covered on both sides either with finely powdered methylen blue, or with a saturated solution of the same, the slices are replaced in their natural positions, the brain case is replaced for half an hour, after which the slices are removed and fixed for a couple of hours with Bethe's ammonium molybdate, washed, hardened for three or four hours in a mixture of 5 parts 1 per cent. platinum chloride, 40 parts formol, and 60 parts water, further treated for a few minutes with platinum chloride in alcohol (1 in 300), and if small enough imbedded in paraffin. The sections should be dehydrated with alcohol containing 0·3 per cent. of platinum chloride, and may be cleared with xylol or bergamot oil and mounted. The stain is stronger and *more complete* than that of the other methylen blue methods.

Catois (*Comptes Rend.*, cxxiv, 1897, p. 124) injects concentrated methylen blue into the body-cavity of FISHES, removes the brain after half an hour, puts slices of it into the same solution for half an hour, and fixes as usual.

LENNHOFF (*Neurol. Zentralb.*, 1910, p. 1) has some complicated methods with polychrome methylen blue and sulpho-cyanide of potassium, or ferricyanide.

834. LENNHOFF'S **Iron Method** (*ibid.*).—Sections put for thirty seconds into 2 c.c. of 15 per cent. solution of tannin with 3 drops of 5 per cent. solution of oxalic acid, rinsed in water, then for a few seconds in 1 per cent. solution of chloride of iron till no further blackening occurs, then washed, dehydrated and mounted in balsam. Axis cylinders black, cells grey.

835. FAJERSTAJN'S **Hæmatoxylin** (*Poln. Arch. Biol. Med. Wiss.*, i, 1901, p. 189).—Sections by the freezing method of material fixed for two to seven days in formol of 5 to 10 per cent. mordanted for five to twenty-four hours in chromic acid of 0·25 to 0·5 per cent., well washed, stained for twenty-four hours in 1 per cent. aqueous hæmatoxylin, and differentiated by the method of PAL.

Other Methods.

836. NABIAS (*C. R. Soc. Biol.*, lvi, 1904, p. 426) treats sections until yellow with solution of 1 grm. iodine and 2 grms. iodide of potassium in 300 of water, washes, treats for a few minutes with 1 per cent. chloride of gold, washes and reduces in anilin or resorcin in water (1 : 100, or less for the latter) and mounts in balsam.

APÁTHY'S **Gold Method** has been given § 371.

GERLACH'S **Bichromate and Gold Process** has been given. § 369.

For a complicated **Gold Method** of RAMÓN Y CAJAL, see *Rev. trim. Micr.*, v, 1900, p. 95; or *Zeit. wiss. Mik.*, xix, 1902, p. 187.

For UPSON'S exceedingly complicated **Gold and Iron and Vanadium Methods** see MERCIER, in *Zeit. wiss. Mik.*, vii, 1891, p. 474; or in his *Coupes du Système Nerveux Central*, p. 234; or *early editions*.

For FAJERSTAJN'S complicated **Silver Method** see *Neurol. Centralb.*, xx, 1901, p. 98; or *Zeit. wiss. Mik.*, xviii, 1901, p. 214.

MAGINI'S **Zinc Chloride Process** (see *Boll. Accad. Med. di Roma*, 1886; *Zeit. wiss. Mik.*, 1888, p. 87, or *early editions*).

MONTI's **Copper Process**, see *Atti. R. Accad. Lincei Roma, Rendic.*, v, 1889, p. 705 ; *Zeit. wiss. Mik.*, vii, 1890, p. 72.

Anthracen Ink (Leonhardi's, obtainable from Grübler) is used in a complicated way by KAPLAN, *Arch. Psychiatr.*, xxxv, 1902, p. 825 (*Zeit. wiss. Mik.*, xix, 1903, p. 510).—STRAHUBER, *Centralb. allg. Path.*, xiii, 1901, p. 422 (*Zeit. wiss. Mik.*, xviii, 1902, p. 482) (pathological). MALLORY's phospho-molybdic hæmatoxylin, see § 271. SAHLI's methods, see § 803.

DONAGGIO's Tin stain, see § 273.

And see also under **Neurofibrils.**

CHAPTER XXXV.

NEUROGLIA, AND SENSE ORGANS.

Neuroglia.

837. Introduction.—Neuroglia cells may be isolated by teasing, and may be stained in many ways (see Ranvier, *Traité*, p. 1063), by osmic acid, nigrosin, carmine, orcein. Iron hæmatoxylin is said to give good results with the lower vertebrates. (I have not found it so.) But by far the best method for the study of the forms and relations both of ependyma cells and astrocytes is the **Bichromate-and-silver Impregnation** of Golgi, the best material being that which has been for not more than two or three days in the osmio-bichromic mixture.

This method, however, does not tinctorially differentiate between neuroglia-cells and nerve-cells, and is of no use for mapping out tracts of neuroglia as a whole. The following methods are intended for this. They either stain neuroglia more or less specifically, leaving other tissues unstained (Weigert), or stain it in a different tone to other tissues. None of them are satisfactory. Weigert's process stains the processes of the cells (his "fibres") intensely, whilst leaving the cell-body unstained; and in consequence, if exclusively followed, may lead to erroneous conclusions.

838. Weigert's **Neuroglia Stain** (Weigert's *Beitr. zur Kenntniss der normalen menschlichen Neuroglia*, Frankfurt-a-M., 1895; and his art. "*Neurogliafärbung*" in *Encycl. Mik. Technik*).—Pieces of *very fresh* tissue of *not more than* half a centimetre in thickness are put for at least four days into 10 per cent. formol. They are then mordanted for four or five days in an incubating stove (or for at least eight days at the temperature of the laboratory) in a solution containing

5 per cent. of neutral acetate of copper, 5 per cent. of acetic acid, and 2½ per cent. of chrome alum, in water. Add the alum to the water, raise to boiling point, and add the acetic acid and the acetate, powdered (or [*Encycl.*, 2nd ed., p. 303] instead of the chrome alum, you may take chromium fluoride, which obviates the necessity of boiling). If preferred, the mordant may be dissolved in the formol solution, so that the hardening and mordanting are done at the same time.

After the mordanting the tissues are washed with water, dehydrated, imbedded in celloidin, and sectioned. The sections (not too thick) are treated for ten minutes with a $\frac{1}{3}$ per cent. solution of permanganate of potash, and well washed in water. They are then treated for two to four hours with a solution of "Chromogen." This is a naphthalin compound prepared by the Hoechst dye manufactory. The solution to be used is prepared as follows: 5 per cent. of "Chromogen" and 5 per cent. of formic acid (*of* 1·20 *sp. gr.*, about four times as strong as the officinal) are dissolved in water, and the solution carefully filtered. To 90 c.c. of the filtrate are added 10 c.c. of 10 per cent. solution of sodium sulphite.

After this bath, the sections are put till next day into a saturated (5 per cent.) solution of Chromogen. (Instead of the Chromogen treatment, you may simply treat the sections with Pal's potassium sulphite, § 783, and the results will be nearly as good.)

They are next carefully washed and stained. This is best done *on the slide*. The stain is a warm-saturated solution of methyl violet in alcohol of 70 to 80 per cent. (to which, after cooling and decanting, there may be added, if desired, 5 per cent. of 5 per cent. aqueous solution of oxalic acid). The sections are treated with this for a few seconds to one minute, and mopped up with blotting-paper, then treated for an instant with saturated solution of iodine in iodide of potassium of 5 per cent. They are then differentiated till clear and light blue with a mixture of anilin and xylol in equal parts. Wash this out thoroughly with pure xylol, and mount in balsam, or, preferably, turpentine colophonium.

Glia fibres and nuclei blue, cytoplasm invisible.

This method *only* gives *good* results with the *human* subject.

Mallory (*Journ. Exper. Med.*, 1897, p. 532) fixes tissues for four days in 10 per cent. solution of formalin, then for four to eight in saturated solution of picric acid (or for the same time in a mixture of the two), then mordants for four to six days at 37° C. in 5 per cent. solution of bichromate of ammonia, makes sections (celloidin) and stains them in Weigert's *fibrin* stain.

Storch (*Virchow's Arch.*, clvii, 1899, p. 127; *Zeit. wiss. Mik.*, xvi, 1900, p. 475), instead of mordanting the material in bulk with the copper fluid, first makes celloidin sections.

Bartel (*Zeit. wiss. Mik.*, xxi, 1904, p. 18) first makes *paraffin* sections and treats them with all the reagents without removing the paraffin, until they have passed the anilin-xylol mixture, which should consist of 1 of anilin to 10 of xylol (or more), and be allowed to act for twelve to twenty-four hours.

Sand takes material fixed as for his neurofibril stain, p. 400, and stains it as Weigert.

See also Aguerre and Krause, *Arch. Mik. Anat.*, clii, 1900, p. 509; and Wimmer, *Zeit. wiss. Mik.*, xxiv, 1907, p. 192.

Rubaschkin (*Arch. mik. Anat.*, lxiv, 1904, p. 577) *injects* centres of *small mammals* with the fixing liquid. To make this, take 100 parts of 2·5 per cent. solution of bichromate of potash and 0·5 to 1 of acetate of copper, boil, and add 2·5 to 3 of glacial acetic acid. To this (which may be kept in stock) add just before use 10 per cent. of formol. Inject warm, and after ten minutes dissect out and harden in the liquid for five to seven days at 35° to 40° C. Dry superficially, put for six to twelve hours in alcohol of 95 per cent., and get into celloidin or paraffin. Stain sections on slide for six to twelve hours in saturated aqueous solution of methyl-violet B; treat for half a minute to a minute with Gram's iodine in iodide of potassium (1 : 200 or 300); differentiate in anilin or clove oil and pass through xylol into balsam. Said to give very sharp results with small mammals.

839. Benda's Methods (*Neurol. Centralb.*, xix, 1900, p. 796, and his art "*Neurogliafärbung*" *Encycl. Mik. Technik.*, ii, p. 308) are as follows: The material is to be fixed with alcohol, and further treated with nitric acid, etc., as directed for *centrosomes*, § 651, and paraffin sections are made and fixed to slides and the paraffin removed. They are then mordanted and stained as directed under (*b*), § 651 and differentiated and mounted as there described.

Glia fibres and nuclei blue, the rest red.

Besides this, Benda also recommends hardening and making paraffin sections as described, then staining with the modified Weigert stain given for central corpuscles under (*a*), § 651; or, staining with Heidenhain's iron hæmatoxylin, and differentiating with 2 per cent. iron-alum or WEIGERT'S borax-ferricyanide mixture.

See also MEVES, *Arch. mik. Anat.*, lxxi, 1908, p. 573.

840. MALLORY'S **Hæmatoxylin Stains** (*Journ. Exper. Med.*, v, 1900, p. 19).—Tissues to be fixed, mordanted, and cut as directed under MALLORY, § 838. The sections are put for a quarter of an hour into 0·5 per cent. solution of permanganate of potash, washed and put for a quarter of an hour into 1 per cent. solution of oxalic acid, well washed and stained for twelve to twenty-four hours or more in Mallory's phosphotungstic hæmatoxylin. Wash, dehydrate in 95 per cent. alcohol, clear with organum oil, mount in xylol-balsam. Axis-cylinders and nerve-cells pink, neuroglia blue. To get a more isolated stain of neuroglia, the sections should be brought for five to twenty minutes, after staining, into a 30 per cent. alcoholic solution of dry sesquichloride of iron. Neuroglia and fibrin blue, the rest colourless.

MALLORY'S phospho-*molybdic* hæmatoxylin may also be taken for the stain, but is less elective.

DA FANO (*Ricerche Lab. Anat. Roma*, xii, 1906, p. 111) fixes in a mixture of 72 vols. of pyridin with 28 of nitric acid of 50 per cent. and stains as Mallory. Or, he fixes in a mixture of 3 vols. of nitrate of pyridin with 1 vol. of osmic acid of 1 per cent., and stains with Benda's alizarin toluidin blue.

FIEANDT (*Arch. mik. Anat.*, lxxvi, 1910, p. 15) describes a very complicated modification of Mallory's phosphotungstic hæmatoxylin method.

ALZHEIMER (quoted from SPIELMEYER'S "*Technik d. mik. Untersuch. d. Nervensystems*," p. 106) fixes in Weigert's mordant (with formol) and stains with Mallory's phosphomolybdic hæmatoxylin.

EISATH (*Arch. Psychiatr. u. Nervenheilk.*, xlviii, 1911, p. 896; *Zeit. wiss. Mik.*, 1913, p. 420) has a highly complicated modification of the same stain, specially for glia granules.

841. ANGLADE and MOREL (*Rev. Neurol.*, ix, 1901, p. 157) harden in a mixture of 3 parts of liquid of FOL (§ 42), with 1 of 7 per cent. sublimate solution, dehydrate with alcohol followed by aceton, make paraffin sections and stain in saturated aqueous solution of *Victoria blue*, heated till it steams, rinse with liquid of Gram (§ 287), differentiate with xylol 1 part, anilin 2 parts, and mount in balsam. Simple, *applicable to lower animals*, and gives very sharp images.

Similarly, in a very complicated way, L'HERMITTE and GUCCIONE, *Semaine Médicale*, xxix, 1909, No. 18, and MERZBACHER, *Journ. f. Psych. u. Neurol.*, xii, 1909, p. 1 (*Zeit. wiss. Mik.*, xxviii, 1911, p. 229).

See also GALESESCU, *C.R. Soc. Biol.*, lxv, 1908, p. 429 (sections mordanted with resorcin and stained with methyl-violet and oxalic acid).

842. **Säurerubin.**—KULTSCHITZKY (*Anat. Anz.*, viii, 1893, p. 357) stains paraffin sections (of material hardened in his copper liquid, § 55), either for five to ten seconds with a mixture of 1 grm. Säurerubin (Rubin S.), 400 c.c. 2 per cent. acetic acid, and 400 c.c. saturated solution of picric acid, or for half an hour in a mixture of 3 to 5 c.c. of the above stain with 100 c.c. of 96 per cent. alcohol and washes out well with alcohol. Glia violet, ganglion cells and axis cylinders reddish.

Insignificant modifications are described by POPOW, *Zeit. wiss. Mik.*, xiii, 1896, p. 358, and BURCHARDT, *La Cellule*, xii, 1897, p. 364.

843. YAMAGIWA (*Virchow's Arch.*, clx, 1900, p. 358) hardens *very small* pieces of tissues for a month or more in liquid of Müller, makes celloidin sections, stains for twelve hours in saturated alcoholic solution of eosin, then for four to six in saturated solution of anilin blue in water, and differentiates in dilute alcohol with a very little caustic potash. Water, alcohol, origanium oil, balsam. Neuroglia red, axis-cylinders blue.

844. ACHÚCARRO (*Bol. Soc. Españ. Biol.*, Madrid, 1911, p. 139; *Zeit. wiss. Mik.*, xxix, 1912, p. 238) puts sections of frozen formol material into cold saturated solution of tannin, warms till vapour is given off, rinses, and puts into 10 c.c. of water with 6 to 8 drops of Bielschowsky's oxide of silver solution (undiluted). As soon as they turn yellow they are put into formol of 10 per cent., and after about 10 minutes washed and mounted.

By mordanting the material with 5 per cent. acetate of copper, or Weigert's mordant, and silvering by Ramón y Cajal's process, he gets a stain of the amœboid cells of the cortex.

*Retina.**

845. **Fixation and Hardening.**—Notwithstanding the *Encycl. mik. Technik.*, 2nd edition, p. 75, I hold that *osmic acid* is by far the best fixing agent. The retina of *small eyes* is best prepared by fixing the entire unopened bulb with osmium

---

* Besides the sources quoted in the text, see SELIGMANN, *Die mikroskopischen Untersuchungsmethoden des Auges*, Berlin, S. Karger (Karlstrasse 13), 1899; GREEF *Anleitung zur Mikr. Untersuch. d. Auges*, Berlin, Hirschwald, 1898; and the Art. "Retina" in *Encycl. mik. Technik.*, 2nd edition, p. 575.

vapour. According to Ranvier (*Traité*, p. 954) you may fix the eye of a triton (without having previously opened the bulb—the sclerotic being very thin) by exposing it for ten minutes to vapour of osmium. Then divide it by an equatorial incision, and put the posterior pole for a few hours into one-third alcohol.

Somewhat larger eyes, such as those of the sheep and calf, may be fixed in solutions without being opened. But it is generally the better practice to make an equatorial incision, and free the posterior hemisphere before putting it into the liquid.

The older practice was to use strong solutions of pure osmic acid alone; but most of the best recent work has been done with chromic mixtures following the osmium.

Dr. Lindsay Johnson tells me that he now gets the best results by suspending the globe over the steam of a 1 per cent. osmic acid solution raised to the temperature at which vapour is seen to be given off (but not to boiling point), for five minutes in the case of human adults, or for one to three minutes in the case of human infants, all monkeys and small mammals, as in them the sclerotics are very thin. As soon as the sclerotic is felt to be firm to the touch, it should be opened by a small nick with a razor just behind the ciliary body; or if the eye be that of an adult, the cornea and lens may be removed. The eye is then put for twelve hours into the mixture, § 44; it is then washed in running water, and suspended in a large volume of 2·5 per cent. bichromate of potash for two days, then passed gradually through successive alcohols, beginning with 20 per cent., and ending with absolute, taking five days from first to last.

Similarly Rochon-Duvigneaud (*Arch. Anat. Micr.*, ix, 1907, p. 317).

Other hardening liquids, however, also give good results, provided that the fixation by the osmic acid has been properly performed: amongst them *liquid of Flemming*, and that of Müller. Formaldehyde mixtures he does *not* recommend.

Leber (*Münch. med. Wochenschr.*, xli, 30, 1894; *Zeit. wiss. Mik.*, xii, 1895, p. 256) advises a solution of formol 1, water 10. After a few days' hardening in this, the eyes may be cut through, it is said, without derangement of the parts. The retina lies flat, and is at least as well preserved as with solution of Müller.

See also HIPPEL (*Arch. f. Ophthalm.*, xlv, 1898, p. 286; *Zeit. wiss. Mik.*, xvi, 1899, p. 79), who finds that formol fixes the lens badly, the retina well, so far at least as the absence of folds from shrinkage is concerned; and HERZOG (*Arch. mik. Anat.*, lx, 1902, p. 517, and *Encycl. mik. Technik.*, p. 75), who also approves of formol, but insists that it should be *acid*, and adds 3 to 5 per cent. of acetic acid.

KOLMER (*Arch. Gesammte Phys.*, cxxix, p. 35), fixes for twelve to twenty-four hours in a mixture of 4 parts saturated solution of bichromate, 4 of formol of 10 per cent., and 1 of acetic acid.

BENDA (*Verh. Ges. Naturf. Ærzte*, lxxi *Vers.*, 1900, p. 459) fixes in nitric acid of 10 per cent., and hardens in liquid of Müller, twenty-four hours in each.

ZÜRN (*Arch. Anat. Phys., Anat. Abth.*, 1902, Supp., p. 106) advises (for mammals) fixing in saturated solution of sublimate in salt solution of 0·6 per cent., with 1 to 1½ per cent. of acetic acid after removing the anterior pole and the vitreous. Wash out in alcohol of 35 per cent. made 5 per cent. stronger each day up to 50 per cent.; then pass on to stronger and cedar oil and paraffin.

846. **Staining.**—For general views I recommend iron-hæmatoxylin, followed by Säurefuchsin or Picro-Säurefuchsin, or preceded by Bordeaux; or Kernschwarz, followed by safranin, or the Ehrlich-Biondi stain.

The **Methylen-blue** *intra-vitam* stain has given valuable results; see the methods of DOGIEL.

But the most important method is the **bichromate-and-silver** impregnation of GOLGI, first applied to this object by TARTUFERI (*Intern. Monatsschr.*, 1887). This author employed the rapid process. So also RAMÓN Y CAJAL (*La Cellule*, ix, 1893, p. 121) with the double-impregnation process, § 815. To avoid the formation of precipitates on the tissues, he covers the retina, before silvering, with a piece of peritoneal membrane, or a thin layer of collodion. Or, better, he *rolls* the retina (*op. cit.*, p. 130). After removing the vitreous, the retina is cut away around the papilla with a punch or fine scalpel, and separated from the choroid. It is then rolled up (after being cut into quadrants or not), so as to form a solid block. This is painted with 2 per cent. celloidin, which is allowed to dry for a few seconds, and the whole is put into the bichromate mixture, and further treated as a solid mass of tissue.

Ramón also employs his neurofibril silver method, see *Intern. Monatsschr. Anat. Phys.*, xxi, 1904, p. 393.

Golgi's **sublimate impregnation** (Cox's form) has also been successfully employed by Krause and Ramón.

The bichromate-and-silver method serves for the study of the fibres of Müller and neuroglia cells, as well as neurones. Weigert's neuroglia stain does not give good results.

Lennox (*Arch. f. Opthalm.*, xxxii, 1; *Zeit. wiss. Mik.*, iii, 1886, p. 408) has used Weigert's hæmatoxylin method.

Kuhnt (*Jen. Zeit. Naturw.*, Bd. xxiv, H. 1, 1889, p. 177) employs Pal's modification. Similarly Schaffer (*Sitzb. Akad. wiss. Wien.*, xcix, 1890), 3, p. 110; *Zeit. wiss. Mik.*, viii, 1891, p. 227). These methods give a differential stain of rods and cones.

For the zonula and ciliary body see Mawas, *Arch. d'Anat. micr.*, xii, 1910, p. 103.

847. Dissociation.—For maceration preparations you may use weak solutions (0·2 to 0·5 per cent.) of osmic acid for fixation, and then macerate in 0·02 per cent. chromic acid (M. Schultze), or in iodised serum (M. Schultze), or in dilute alcohol (Landolt), or in Müller's solution, or (Ranvier, *Traité*, p. 957) in pure water, for two or three days. Thin (*Journ. of Anat.*, 1879, p. 139) obtained very good results by fixing for thirty-six to forty-eight hours in one-third alcohol, or in 25 per cent. alcohol, and then staining and teasing.

Schiefferdecker macerates fresh retina for several days in the methyl mixture, § 543.

Krause (*Intern. Monatsschr. Anat.*, 1884, p. 225) recommends treatment for several days with 10 per cent. chloral hydrate solution; the rods and cones are well preserved.

Inner Ear.

848. Inner Ear, Dissection.—For the dissection of the human ear see Politzer, "Die anatomische u. histologische Zergliederung d. menschlichen Gehörorganes," Stuttgart (Enke), 1889 (*Zeit. wiss. Mik.*, vii, 1890, p. 364). Amongst the lower mammalia, the *guinea-pig* is a favourable subject, as here (as with some other rodents) the cochlea projects freely into the cavity of the bulla, and may be easily removed with a scalpel and brought into a fixing liquid, and opened therein. With fishes and amphibia also the membranous labyrinth may easily be got away.

849. Preparation.—Schwalbe (*Beitr. z. Phys.*, 1887; *Zeit. wiss. Mik.*, iv, 1887, p. 90).—Fix (cochlea of guinea-pig) for eight to ten hours in "Flemming," wash in water, decalcify (twenty-four hours is enough) in 1 per cent. hydrochloric acid, wash the acid out, dehydrate, and imbed in paraffin.

Prenant (*Intern. Monatssch. Anat.*, ix, 1892, p. 28).—Open the cochlea in solution of Flemming or of Hermann, and fix therein for four to five hours. Avoid decalcification as far as possible, but if necessary take 1 per cent. palladium chloride. Make paraffin sections.

Isolation preparations of the stria vascularis may be made by putting a cochlea for a day into 1 per cent. solution of osmic acid, then for four to five days into 0·1 per cent. solution; the stria may then be got away whole.

Katz (*Zeit. wiss. Mik.*, xxv, 1908, p. 111) fixes the inner ear, opened, for one or two hours in 30 c.c. of 0·5 per cent. osmic acid with 5 drops of acetic acid, then adds 10 drops of acetic acid and 60 c.c. of chromic acid (or platinum chloride) of 0·5 per cent. and leaves it for four days therein. He then rinses, puts for twelve to twenty-four hours into pyroligneous acid or pyrogallol or tannin solution, decalcifies (not necessary for mice) in 200 parts of water with 1 of chromic acid and 4 to 10 of nitric or hydrochloric acid, and imbeds in celloidin or sometimes paraffin.

Similarly Wittmaack, see § 799.

Bielschowski and Bruehl (*Arch. mik. Anat.*, lxxi, 1907, p. 27) fix the petrous in formol of 20 per cent., decalcify it in nitric acid of 5 per cent., wash this out, and put back for a few days into the formol, cut by the freezing method, and silver by the neurofibril method § 770 (24 hours in nitrate of 4 per cent., but only a few minutes in the oxide bath).

Similarly Mullenix, *Bull. Mus. Comp. Zool. Harvard Coll.*, liii, 1909, p. 215.

Stein (*Anat. Anz.*, xvii, 1900, p. 398) decalcifies in celloidin by the method of Rousseau. So also Kishi (*Arch. mik. Anat.*, lix, 1901, p. 173).

For *staining*, Ranvier (*Traité*, p. 991) employs his gold and formic-acid method.

The bichromate-and-silver method of Golgi may be employed with *fœtal or new-born* subjects. The *methylen blue intra vitam* method has given good results. For the higher vertebrates the injection method should be employed. The *Encycl. mik. Technik*, i, p. 511, recommends injection of 1 c.c.

of 0·5 per cent. to 1 per cent. solution every five minutes through the vena femoralis, until the death of the animal. The cochlea then to be got out, exposed to the air for 15 or 30 minutes, and fixed for some hours (overnight) in 10 per cent. ammonium molybdate with a little osmic acid. Then decalcified in trichloracetic acid of 5 per cent. with a trace of platinum chloride, washing for 24 hours and got into paraffin.

For fishes and amphibia the immersion method will suffice.

850. Other Methods.—WALDEYER, Stricker's *Handb.*, p. 958 (decalcification either in 0·001 per cent. palladium chloride containing 10 per cent. of HCl, or in chromic acid of 0·25 to 1 per cent.).

URBAN PRITCHARD (*Journ. Roy. Mic. Soc.*, 1876, p. 211).—Decalcification in 1 per cent. nitric acid.

LAVDOWSKY (*Arch. mik. Anat.*, 1876, p. 497).—Fresh tissues (from the cochlea) are treated with 1 per cent. solution of silver nitrate, then washed for ten minutes in water containing a few drops of 0·5 or 1 per cent. osmic acid solution, and mounted in glycerin.

MAX FLESCH (*Arch. mik. Anat.*, 1878, p. 300); TAFANI (*Arch. Ital. de Biol.*, vi, p. 207); EICHLER, *Abh. math-phys. Cl. Sächs. Ges. Wiss.*, xviii, 1892, p. 311; *Zeit. wiss. Mik.*, ix, 1893, p. 380 (injection of blood-vessels of the labyrinth); SIEBENMANN, *Die Blutgefässe im Labyrinthe des menschlichen Ohres*, Wiesbaden, Bergmann, 1894; *Zeit. wiss. Mik.*, xi, 1894, p. 386; GRAY, *Journ. Anat. Phys.*, 1903, p. 379; SCOTT, *ibid.*, xliii, 1909, p. 329.

851. Olfactive Nerve-endings, Tactile Corpuscles, etc.—Besides the *gold method*, Chapter XVII, and the *methylen-blue method*, Chapter XVI, the rapid *bichromate-and-silver method* of GOLGI should be employed, and for the olfactive mucosa gives the best results; see VAN GEHUCHTEN, *La Cellule*, vi, 1890, p. 405. For *intra-epidermic nerve-endings*, besides the methods given Chapter XXVII, the GOLGI method should be employed. According to VAN GEHUCHTEN (*La Cellule*, ix, 1893, p. 319) it gives much better results than gold methods. He uses the rapid process. For *tactile corpuscles*, etc., besides the methods given § 661 and 662, see RAMÓN Y CAJAL's neurofibril methods.

CHAPTER XXXVI.

METHODS FOR INVERTEBRATES.

Tunicata.

852. Fixation of Tunicata.—A method of Lo Bianco[*] for killing simple Ascidians in an extended state has been given, § 25. Some forms, such as *Clavellina, Perophora, Phallusia, Molgula, Cynthia*, etc., should first be narcotised by treatment for from three to twelve hours with chloral hydrate (1 : 1000 in sea water), then killed in a mixture containing chromic acid of 1 per cent. 10 parts, acetic acid of 50 per cent. 100 parts, and finally hardened in 1 per cent. chromic acid.

The compound Ascidians with contractile zooids may be left in clean sea water till the zooids have become fully extended, then fixed by van Beneden's acetic acid process, § 84 (steel instruments being avoided for manipulating them). I strongly recommend this process.

S. Lo Bianco recommends for this group the chloral hydrate process, followed by fixation with sublimate or chromo-acetic acid.

Caullery (*Bull. Sc. France Belg.*, xxvii, 1895, p. 5) first stupefies the animals with cocaine (Lahille, a few drops of 5 per cent. solution to 30 c.c. of sea water), then fixes in liquid of Flemming or acetic acid.

Most small *pelagic* Tunicates are very easily fixed with osmic acid or acid sublimate solution.

I have found the acetic acid process very good for *Pyrosoma*. Lo Bianco puts them for a quarter of an hour into 50 per cent. alcohol containing 5 per cent. of hydrochloric acid, then into successive alcohols, beginning with 60 per cent. He kills the hard forms of *Salpa* with acetic acid of 10 per cent., the semi-hard ones with 1 per cent. chromic acid containing 5 per cent. acetic acid, the soft ones with 1 per cent. chromic acid containing $\frac{1}{50}$ per cent. osmic acid, or 10 parts of 1 per cent. chromic acid, with 1 of formol and 9 of sea-water, Doliolidæ with sublimate, or the above osmic mixture, or a mixture of 10 parts 10 per cent. solution of sulphate of copper with 1 part concentrated sublimate solution, or the formol mixture.

---

[*] References to methods of Lo Bianco in this Chapter are all to his paper in *Mitth. Zool. Stat. Neapel*, ix, 1890, p. 435.

Molluscoida.

853. Bryozoa.—For some methods of killing and fixing see §§ 13, 20, and 21. S. Lo Bianco employs for *Pedicellina* and *Loxosoma* the chloral hydrate method, fixing with sublimate. For *Flustra, Cellepora, Bugula, Zoobothrium,* he employs the alcohol method of Eisig, § 18. For *Cristatella* see §§ 16, 20. See also Braun, § 907.

Conser (*Trans. Amer. Mic. Soc.*, xvii, 1896, p. 310) kills the fresh-water forms with cocaine, puts them for an hour into 1 per cent. chromic acid, and passes through water into alcohol, etc.

Similarly Calvet (*Hist. Nat. Bryozoaires,* Montpellier, 1900, p. 15), for marine forms.

Zschiesche (*Zool. Jahrb.*, xxviii, 1909, p. 6) fixes larvæ of *Alcyonidium* (settled down on a layer of celloidin or paraffin) with 8 parts of sublimate and 2 of acetic acid to 90 of sea water, for 25 to 30 minutes.

854. Brachiopoda.—Lo Bianco kills small animals in 70 per cent. alcohol, larger ones being first narcotised with alcohol and sea water.

Blochmann (*Untersuch. fein. Bau Brachiopoden,* Jena, 1892, p. 5) fixes principally with sublimate, macerates by the Hertwigs' method, § 534, decalcifies with 1 per cent. chromic acid (for thick shells add a little hydrochloric or nitric acid), or with nitric acid in alcohol of 50 to 70 per cent., and imbeds in paraffin or celloidin.

See also Ekman, *Zeit. wiss. Zool.*, lxii, 1896, p. 172.

Mollusca.

855. Fixation.—Lo Bianco narcotises Lamellibranchs for six to ten hours or more with alcohol, § 18, and then kills them.

List (*Fauna Flora Golf. Neapel*, xxvii, 1902, p. 292) narcotises Mytilidæ with 2 per cent. of cocaine in sea water, and (for preservation of cilia) fixes in sea water, with 10 per cent. of formol.

Lo Bianco advises that Prosobranchiata, and, amongst the Heteropoda, Atlantidæ, be narcotised with 70 per cent. alcohol, § 18. For Opisthobranchiata I recommend sudden killing with liquid of Perényi, or the acetic method, § 852. *Aplysia* may first be narcotised by subcutaneous injection of about 1 c.c. of a 5 to 10 per cent. solution of hydrochlorate of cocaine (Robert, *Bull. Scient. de la France*, etc., 1890, p. 449; *Zeit. wiss. mik.*, ix, 1892, p. 216), or (Schönlein, *Zeit. Biol.*, xxx, 1893, p. 187) 1 c.c. of 4 per cent. solution of Pelletierin. For Lo Bianco's various methods see the original, p. 467.

For Pteropoda in general, liquid of Perényi. *Creseis* is a difficult form. Lo Bianco advises the alcohol method, § 18. For the Gymnosomata he narcotises with 0·1 per cent. chloral hydrate.

For terrestrial Gastropods see §§ 23 and 26. Marchi (*Arch. mik. Anat.*, 1867, p. 204) gets rid of the mucus of the integument of Limax, which may be an obstacle to preparation, by putting the living animal into moderately concentrated salt solution, in which it throws off its mucus and dies in a few hours.

Lang (*Anat. Hefte*, 1902, p. 84) puts *Helix* into water with enough chromic acid to make it of a Rhine-wine colour, with an air-tight cover to the vessel, and when the animals are extruded injects into them a quarter to a half of a Pravaz syringe of 1 per cent. cocain, and after five to fifteen minutes dissects and fixes.

Heymans (*Bull. Acad. Belg.*, xxxii, 1896, p. 578) injects ethyl bromide under the skin of Cephalopoda.

Lo Bianco uses for fixing them his chromo-acetic acid, No. 1 (§ 39), with a double quantity of acetic acid, for twenty-four hours.

856. Liver of Mollusca.—Enriques (*Mitth. Zool. Stat. Neapel.*, xv, 1901, p. 289) fixes the liver of *Octopus and Sepia* with sublimate. For *Aplysia* (especially in summer) alcohol, formol, and chromic mixtures are counter-indicated, on account of the carbohydrates in the cell. Sublimate is best.

857. Nervous System of Pulmonata.—B. de NABIAS (*Act. Soc. Linn. Bordeaux*, 1894; *Rech. Hist. centres nerveux des Gastéropodes*, 1894, p. 23) opens the animals and fixes the ganglia for one hour in a mixture of 6 parts glacial acetic acid to 100 of 90 per cent. alcohol, or for fifteen to twenty minutes in 5 per cent. sublimate with 5 per cent. acetic acid. He stains in bulk, with Renaut's hæmatoxylic eosin, or R. Heidenhain's hæmatoxylin, or a copper hæmatoxylin of Viallanes, § 874, and imbeds in paraffin. He also stains by the rapid method of GOLGI, imbedding, however, the ganglia in celloidin directly after the hardening in osmic acid and bichromate, and treating the sections with the silver (p. 34). He stains with methylen blue by treating the ganglia *in situ* for twelve to twenty-four hours with a 1 per cent. solution.

DREYER (*Zeit. wiss. Zool.*, xcvi, 1910, p. 380) narcotises Nudibranchs with cocain, and for studying the nerves fixes them with MAYER's picro-formol, puts for a week into a mixture of 1 grm. of iron alum with 2 c.c. of formol and 40 of water, makes sections and stains with iron hæmatoxylin.

See also, for nerve-cells, McCLURE, *Zool. Jahrb.*, 1898, p. 17 (MANN's methyl blue and eosin, or BENDA's safranin and Lichtgrün), and LEGENDRE, *Arch. mic. Anat.*, x, 1909, p. 312.

858. Eyes of Gastropoda (FLEMMING, *Arch. mik. Anat.*, 1870, p. 441).—To obtain the excision of an exserted eye, make a rapid cut at the base of the peduncle, and throw the organ into very dilute chromic acid, or 4 per cent. bichromate; after a short time it will evaginate, and remain as completely erect as if alive. Harden in 1 per cent. osmic acid, in alcohol, or in bichromate.

SMITH (*Bull. Mus. Comp. Zool. Harvard*, xlviii, 1906, p. 238) macerates eyes for at least two days in 9 parts of water with 1 of weak mixure of Flemming, followed by glycerin of 10 per cent. He bleaches them (in sections) with nitric acid and chlorate of potash.

859. Eyes of Cephalopoda and Heteropoda (GRENACHER, *Abh. naturf. Ges. Halle-a.-S.*, Bd. xvi, 1896, p. 213).—Depigment with hydrochloric acid (in preference to nitric acid). The mixture § 582 may also be used. If you stain with borax-carmine and wash out in this mixture, the pigment will be found to be removed quicker than the stain is washed out.

Lenhossék (*Zeit. wiss. Zool.*, lviii, 1894, p. 636; *Arch. mik. Anat.*, xlvii, 1896, p. 45) applies the method of Golgi to the eyes of Cephalopods.

Similarly Kopsch (*Anat. Anz.*, xi, 1895, p. 362), but using formol instead of the osmic acid.

Hesse (*Zeit. wiss. Zool.*, lxviii, 1900, p. 418) fixes eyes of Heteropoda with one of formol to 4 of water, and (p. 456) bleaches those of Cephalopoda by the methods of Grenacher and that of Jander, § 583.

See also Merton, *ibid.*, lxxix, 1905, p. 326.

860. **Eyes of Lamellibranchiata.**—See Patten, *Mitth. Zool. Stat. Neapel*, vi, 1886, p. 733, and Rawitz, *Jena. Zeit. Naturw.*, xxii, 1888, p. 115, and xxiv, 1890, p. 579 (bleaches with caustic soda); see § 584. Hesse (*op. cit.*, last §, p. 380) employs the method of Jander for *Arca*. He fixes the eye of *Pecten* in 10 per cent. formol for five minutes, followed by sublimate or picro-nitric acid.

861. **Shell.**—Sections of non-decalcified shell are easily obtained by the usual methods of grinding, or, which is often a better plan, by the methods of v. Koch or Ehrenbaum. Moseley (*Quart. Journ. Mic. Sci.* [2], xxv, 1885, p. 40) decalcifies with nitric acid of 3 to 4 per cent. and then makes sections. This method serves for the study of the eyes of Chitonidæ.

862. **Injection of Acephala** (Flemming, *Arch. mik. Anat.*, 1878, p. 252).—To kill the animals freeze them in a salt-and-ice mixture, and throw them for half an hour into lukewarm water. They will be found dead, and the injection-pipe may be tied in the heart, and the entire animal filled and covered up with plaster-of-Paris, which serves to occlude cut vessels that it is not possible to tie. As soon as the plaster has hardened the injection may be proceeded with. See also Dewitz, *Anleit. zur Anfert. zootom. Präp.*, Berlin, 1886, p. 44 (*Anodonta*) and p. 52 (*Helix*).

Dakin (*Liverpool Mar. Biol. Comm.*, xvii, 1909, p. 76) narcotises by adding alcohol and glycerin for eighteen to twenty-four hours, puts for half an hour into formol of 5 per cent., and injects from a branchial vessel.

Mozejko (*Zeit. wiss. Mik.*, xxvi, 1909, p. 353, and 1910, p. 542) puts for half an hour into water at 40° to 50° C., removes the shell, and injects carmine by auto-injection through the heart. For occluding vessels he takes cotton-wool soaked with gelatin and plaster-of-Paris. He takes for a vaso-dilator a saturated solution of *peptonum siccum*.

863. Maceration Methods for Epithelium. — Engelmann (*Pflüger's Arch.*, xxiii, 1880, p. 505) macerates the intestine of *Cyclas* in osmic acid of 0·2 per cent. (after having warmed the animal for a short time to 45° to 50° C.), or in concentrated boracic acid solution.

Cilia.—The entire intra-cellular fibre apparatus may be *isolated* by teasing fresh epithelium from the intestine of a Lamellibranch (*e. g. Anodonta*) in either bichromate of potash of 4 per cent. or salt solution of 10 per cent. To get good views of the apparatus *in situ* in the body of the cell, macerate for not more than an hour in concentrated solution of boracic or salicylic acid. Very dilute osmic acid (*e. g.* 0·1 per cent.) gives also good results. The "lateral cells" of the gills are best treated with strong boracic acid solution (five parts cold saturated aqueous solution to one part water).

Bela Haller's **Mixture**, see § 540; Brock's **Medium**, § 531; Möbius's **Media**, § 535; the second of these is much recommended by Drost (*Morphol. Jahrb.*, xii, 1866, p. 163) for *Cardium* and *Mya*.

Patten (*Mitth. Zool. Stat. Neapel*, vi, 1886, p. 736) takes sulphuric acid, 40 drops to 50 grm. of water. Entire molluscs, without the shell, may be kept in it for months.

Bernard (*Ann. Sci. Nat.*, ix, 1890, p. 191) macerates the mantle of Prosobranchs in a mixture of one part each of glycerin and acetic acid, two parts each of 90 per cent. alcohol and 0·1 per cent. chromic acid and forty parts water, which acts in from a quarter of an hour to three hours. He also (pp. 102, 306) uses a weak solution of chloride of ruthenium, especially for nerve-tracts, mucus-cells and cilia. Alcohol material may be macerated in a mixture of one part glycerin, two of acetic acid and forty of water.

864. Mucus Glands.—Racovitza (*Arch. Zool. expér.* [3], ii, 1894, p. 8) studies these in Nudibranchs (and Annelids) by killing with acetic acid, staining *in toto* with methyl green dissolved in liquid of Ripart and Petit, and after three to six days, when only the glands show the stain, examining in mixture of equal parts of glycerin and the liquid.

Arthropoda.

865. General Methods for Anthropoda.—As general methods for the study of chitinous structures, the methods worked out by Paul Mayer (see §§ 8 and 96) are excellent. It is, at all events, absolutely necessary, in the preparation of *entire organisms or unopened organs*, that all processes of fixation, washing and staining should be done with fluids possessing great penetrating power. Hence picric acid combinations should in general be used for fixing, and alcoholic fluids for washing and staining. *Concentrated* picro-sulphuric acid (or *picro-nitric*) is the most generally useful fixative, and 70 per cent. alcohol is the most useful strength for washing out. *Alcoholic* picro-sulphuric acid may be indicated for fixing in some cases.

But if the animals or organs can first be properly opened, the usual methods may be employed.

866. Crustacea.—Some forms are very satisfactorily fixed with sublimate. Such are the Copepoda and the larvæ of Decapoda. It is sometimes indicated to use the sublimate in *alcoholic* solution. Some Copepoda, however (*Copilia, Sapphirina*), are better preserved by means of weak osmic acid, and so are the Ostracoda. In many cases the osmic acid will produce a sufficient differentiation of the tissues, so that further staining may be dispensed with; so for *Copilia* and *Phyllosoma*. The pyrogallic process (§ 374) may be useful. Giesbrecht takes for marine Copepods a concentrated solution of picric acid in sea water, to which a little osmic and acetic acid may be added. For fresh-water forms, Zacharias (*Zool. Anz.*, xxii, 1899, p. 72) takes chromo-acetic acid.

Giesbrecht fixes larvæ of Stomatopoda for 5 to 10 minutes in formol of 10 per cent. warmed to 40° or 50° C., opens them in sea water and puts for 1½ to 2½ hours into formol 1 part and sea water 5 parts, and brings into alcohol of 70 per cent.

Stappers (*La Cellule*, xxv, 1909, p. 356) fixes Sympoda in Gilson's copper formol, § 115, or in Hornell's mixture of 100 parts of 5 per cent. formol with 40 of alcohol; and for softening the chitin puts for 12 to 36 hours into 3 per cent. solution of sublimate with 5 per cent. of nitric acid.

Nettovitch (*Arb. z. Inst. Wien*, xiii, 1900, p. 3) fixes *Argulus* with liquid of Tellyesniczky, § 52, warmed to 50° C.

For Fischel's *intra-vitam* stain of Cladocera with alizarin etc., see § 208.

867. Tracheata.—Kenyon (*Tufts Coll. Stud.*, No. 4, 1896, p. 80) fixes Pauropoda in Carnoy's acetic alcohol and chloroform, § 85, cuts them in two for staining, etc., and imbeds in celloidin followed by paraffin.

Hennings (*Zeit. wiss. Mik.*, xvii, 1900, p. 311) takes—Nitric acid 16 parts, chromic acid of 0·5 per cent. 16 parts, sublimate saturated in 60 per cent. alcohol 24 parts, picric acid saturated in water 12, and absolute alcohol 42, fixes for twelve to twenty-four hours, and washes out with iodine alcohol. He says that this mixture not only fixes, but softens chitin enough to allow of paraffin sections being made through hard parts.

Hamann (*Sitz. Naturw. Freunde Berlin*, 1897, p. 2) fixes small Tracheata in 10 per cent. formol and finds the chitin sufficiently soft for sections to be made.

Van Leeuwen (*Zool. Anz.*, xxxii, 1907, p. 318) takes for larvæ of Hexapoda 12 parts of 1 per cent. solution of picric acid in absolute alcohol, 2 of chloroform, 2 of formol, and 1 of acetic acid.

Hollande (*Arch. d'Anat. mic.*, xiii, 1911, p. 171), takes 12 parts of saturated solution of picric acid in formol of 40 per cent., 54 of absolute alcohol, 3 of benzene, and 1 of nitric acid, and finds that this fixes quickly enough not to make chitin too hard.

Nuttall, Cooper and Robinson (*Parasitology*, 1908, i, p. 163), fix for a few minutes in hot picrosulphuric acid.

868. Methods for Clearing and Softening Chitin.—The methods of Looss have been described § 553, those of Hennings and Hamann last §.

List (*Zeit. wiss. Mik.*, 1886, p. 212) treats Coccidæ (after hardening) for eighteen to twenty-four hours with *eau de Javelle*, diluted with four volumes of water. After washing out they may be imbedded in paraffin, and good sections obtained.

Saling (*Dissert. Marburg.* 1906, p. 11) boils larvæ of *Tenebrio* for some minutes in *eau de Labarraque*, the heat serving to fix the soft parts, which in successful cases are well preserved. Wash out with warm water, then alcohol.

Sazepin's method for antennæ of Chilognatha (*Mém. Acad. Imp. St. Pétersb.*, xxxii, 1884, pp. 11, 12) consists in steeping antennæ (that have been dehydrated with alcohol) for twenty-four hours in chloroform containing a drop of fuming nitric acid (shake occasionally).

Bethe (*Zool. Jahrb.*, viii, 1895, p. 544) puts *telsons of Mysis* for eight to fourteen days into 40 per cent. alcohol, to which nitric acid is gradually added, so that by the end of that time they have been brought into alcohol containing 20 per cent. of the acid. This softens the chitin, and somewhat breaks down the structure of the otolith, so that good sections through it are occasionally obtained.

Similarly Herbst, *Arch. Entwickelungsmech* ix, 1899, p. 291.

See also the depigmentation processes, §§ 575—584.

869. Test for Chitin (Zander, *Pflüger's Arch.*, lxvi, 1897, p. 545).—Treat for a short time with a drop of freshly prepared solution of iodine in iodide of potassium and add a drop of concentrated chloride of zinc. This is then removed with water as far as possible, and the violet reaction is obtained.

See also Wester, *Zool. Jahrb.*, *Abth. Syst.*, xxviii, 1910, p. 531.

870. Bethe's Stain for Chitin (*loc. cit.*, § 868).—Sections are put for three or four minutes into a freshly prepared 10 per cent. solution of anilin hydrochloride, to which has been added one drop of hydrochloric acid for every 10 c.c. They are then rinsed in water, and the slide is put with the sections downwards into 10 per cent. solution of bichromate of potash. The stain is at first green, but becomes blue in tap water or alcohol containing ammonia.

Mayer simply uses a solution of pyrogallol in alcohol or glycerin; and Hofmann (*Zeit. wiss. Zool.*, lxxxix, 1908, p. 684) puts for a day or more into raw pyroligneous acid.

871. Tracheæ may be studied by the Golgi bichromate and

silver process. MARTIN (*C.R. Soc. Philomath.*, 1893, p. 3) injects them with *indigo white* (through the body cavity), and puts into hot water from which the air has been expelled by boiling. Tracheæ blue.

872. Brain of Bees.—KENYON (*Journ. Comp. Neurol.*, vi, 1896, p. 137; *Journ. Roy. Mic. Soc.*, 1897, p. 80) treats by the GOLGI process (seldom successful), or hardens in a mixture of one part formol and two of 5 per cent. sulphate of copper, followed by staining in Mallory's phospho-molybdic hæmatoxylin.

JONESCU (*Jena. Zeit.*, xlv, 1909, p. 111) has employed the silver methods of Ramón y Cajal and Bielschowsky and Wolff.

873. Ventral Cord.—FLOYD (*Mark. Anniv. vol.* 1904, p. 355) fixes the ganglia of *Periplaneta* for eighty minutes with vapour of formol, and brings into alcohol.

See also BINET, *Journ. Anat. Phys.*, xxx, 1894, p. 469.

874. Eyes of Arthropods.—For the methods of LANKESTER and BOURNE (*Quart. Journ. Mic. Sci.*, 1883, p. 180: *Limulus*); HICKSON (*ibid.*, 1885, p. 243: *Musca*); PARKER (*Bull. Mus. Harvard Coll.*, xx, 1890, p. 1; *Zeit. wiss. Mik.*, viii, 1891, p. 82: *Homarus*) see *early editions.*

PARKER (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 1) also applies the methylen blue method to the retina and optic ganglia in Decapods, especially in *Astacus*. He injects 0·1 c.c. of a 0·2 per cent. solution into the ventral sinus. After twelve to fifteen hours the animals are killed, the ganglia quickly dissected out, and the stain fixed as described, § 344.

For his method for eyes of *Scorpions* see § 583.

For the methods of PURCELL for the eyes of *Phalangida* see *Zeit. wiss. Zool.*, lviii, 1894, p. 1. He has the following stain. The cephalothorax is removed and brought for twenty minutes into 50 per cent. alcohol warmed to 45° or 50° C., and saturated with picric acid. The pigment dissolves in this solution and stains the nuclei and some other parts of the rhabdoms, so that no further stain is required.

HENNINGS (*Zeit. wiss. Mik.*, xvii, 1900, p. 326) depigments sections by putting then for ten minutes (Musca) to twelve hours (Myriopoda) into a mixture of 2 parts of 80 per cent. alcohol with one of glycerin and 2 per cent. of nitric acid, best kept at 35° C. The elements are well preserved.

WIDMANN (*Zeit. wiss. Zool.*, xc, 1908, p. 260) makes the lens of Arachnida fit for sectioning by putting for a day or so into alcohol with 10 to 15 per cent. of nitric acid; and bleaches sections with 1 part of chlorine water to 2 of alcohol.

See also ROSENSTADT, *Arch. mik. Anat.*, xlvii, 1896, p. 478; VIALLANES, *Ann. Sci. nat.*, xiii, 1892, p. 354; and DIETRICH, *Zeit. wiss. Zool.*, xcii, 1909, p. 465 (fixes in alcoholic formol, and bleaches with dilute *aqua regia*).

875. Injections (Arachnida and Crustacea especially).—AIMÉ SCHNEIDER (*Tablettes Zool.*, ii, 1892, p. 123) recommends lithographic Indian ink, the animals being narcotised with chloroform, then injected and thrown into strong alcohol. Similary CAUSARD (*Bull. Sc. France Belg.*, xxix, 1896, p. 16).

876. Arctiscoida (DOYÈRE, *Arch. mik. Anat.*, 1865, p. 105).—Examination of living animals after partial asphyxiation in boiled water. See *early editions.*

Vermes.

877. Chætopoda: Cleansing Intestine.—KÜKENTHAL (*Journ. Roy. Mic. Soc*, 1888, p. 1044) puts *Lumbricus* into a glass vessel filled with bits of moistened blotting-paper. They gradually evacuate the earthy particles from the gut, and fill it instead with paper.

VOGT and YUNG (*Traité d'Anat. Comp. Prat.*, v) recommend coffee-grounds instead of paper, as they cut better after imbedding.

JOEST (*Arch. Entwicklungsmech.*, v, 1897, p. 425) simply keeps the worms for a few days in moist linen, and finds the gut empty.

PEARL (*Journ. appl. Mic.*, iii, 1901, p. 680) injects alcohol of 6 per cent. through the gut of narcotised worms.

878. Chætopoda: Fixation.—*Lumbricus* may be anæsthetised by putting the animals into water with a few drops of chloroform. PERRIER puts them into water in a shallow dish, sets up a watch-glass with chloroform in the corner of it, and covers the whole.

Cerfontaine (*Arch. de Biol.*, x, 1890, p. 327) injects interstitially about 2 c.c. of a 1 : 500 solution of curare.

Jaquet (*Bib. Anat.*, iii, 1895, p. 32) kills *Lumbricus* in extension in 1 part of nitric acid to 125 of water.

Collin (*Zeit. wiss. Zool.*, xlvi, 1888, p. 474) puts *Criodrilus lacuum* into a closed vessel with a little water, and hangs up in it a strip of blotting-paper soaked in chloroform. Kükenthal (*Die mik. Technik*, 1885 ; *Zeit. wiss. Mik.*, 1886, p. 61) puts Annelids into a glass cylinder filled with water to the height of 10 centimetres, and then pours 70 per cent. alcohol to a depth of one to two centimeters on to the water. For Opheliadæ he also employs 0·1 per cent. of chloral hydrate in sea water.

Many marine Chætopoda may be successfully narcotised (Lo Bianco) in sea water containing 5 per cent. of alcohol, or by means of the mixture § 18.

The *Polychæta sedentaria* may sometimes be satisfactorily fixed by bringing them rapidy into corrosive sublimate. Cold, not hot, solutions should be taken, as heat frequently shrivels up the branchiæ. *Eunice* and *Onuphis* may be treated in the same way.

Lo Bianco advises killing Chætopteridæ, Sternaspidæ, *Spirographis*, *Protula*, by putting them for half an hour into 1 per cent. chromic acid. Some of the *sedentaria* may be got protruded from their tubes by leaving them for some hours in 0·1 per cent. chloral hydrate in sea water.

For Eisig's methods for Capitellidæ see *Fauna u. Flora Golf. Neapel*, xvi, 1887, p. 295.

See also § 14 (lemon juice), and the methods §§ 20 to 26, 39 and 49.

879. Blood-vessels of Annelids (Kükenthal, *Zeit. wiss. Mik.*, 1886, p. 61).—The animals should be laid open and put for two or three hours into *aqua regia* (4 parts of nitric acid to 2 of hydrochloric acid). Vessels black, on a yellow ground.

Bergh (*Anat. Hefte*, xlv, 1900, p. 392, and xlix, 1900, p. 599) puts small Annelids for a week or more into equal parts of 1 per cent. nitric acid and 1 per cent. nitrate of silver, or into 50 parts of nitrate, 25 of formic acid, and 25 of water, dissects out the organs and exposes to light. Marine forms may be treated by Harmer's process.

880. Nerves of Annelids.—The methylen blue method and the bichromate of silver method of Golgi (the *rapid* method). For the latter see v. LENHOSSÉK (*Arch. mik. Anat.*, xxxix, p. 102).

LANGDON (*Journ. Comp. Neur.*, x, 1900, p. 4) injects strong solution of methylen blue into the body cavity of *Nereis*, and puts the animal for some hours into sea-water in the dark, fixes the stain by Bethe's method, and makes paraffin sections.

See also M. LEWIS, *Anat. Anz.*, xii, 1896, p. 292; ATHESON, *ibid.*, xvi, 1899, p. 497; and the methods of APÁTHY §§ 342, 368, 371, and 773.

881. Hirudinea.—For the methods of killing see those given for *Lumbricus* in § 878, also §§ 20 to 26, and 49.

WHITMAN (*Meth. in mic. Anat*, p. 27) recommends that they be killed with sublimate.

I have obtained better results myself by narcotising with carbonic acid (§ 26), and fixing with liquid of Flemming. I have also found that *lemon juice* kills them in a state of very fair extension.

APÁTHY succeeds with alcohol of 40 per cent.

GRAF (*Jen. Zeit.*, 1893, p. 165) has obtained good results by narcotising with decoction of tobacco.

882. Injection.—WHITMAN (*Amer. Natural.*, 1886, p. 318) states that very perfect natural injections may often be obtained from leeches that have been hardened in weak chromic acid or other chromic liquid.

JACQUET (*Mitth. Zool. Stat. Neapel*, 1885, p. 298), for artificial injections, puts leeches into water with a very small quantity of chloroform, and allows them to remain a day or two in the water before injecting them.

883. Nervous System.—Impregnation with gold. BRISTOL (*Journ. of Morph.*, xv, 1898, p. 17) kills in formic acid of 15 to 20 per cent., puts for twenty-five minutes into 1 per cent. gold chloride, reduces in formic acid of 1 per cent. (twelve to eighteen hours), and imbeds in paraffin. See also §§ 342, 368, 371 and 773.

884. Nephridia.—SHEARER (*Quart. Journ. Micr. Sci.*, lv, 1910, p. 288) stains *Histriobdella* intra vitam with very weak solution of *Methyl* blue, which allows the course of the nephridia to be made out.

885. **Gephyrea.**—VOGT and YUNG (*Anat. Comp. Prat.*, p. 373) direct that *Siphunculus nudus* be kept for some days in perfectly clean basins of sea water, changed every day, in order that the intestine of the animals may be got free from sand, and then anæsthetised with chloroform.

WARD (*Bull. Mus. Comp. Zool., Cambridge, Harvard Coll.*, xxi, 3, p. 144) puts them into a shallow dish with sea water and pours 5 per cent. alcohol in a thin film on to the surface of the water, and as soon as they make no contractions on being stimulated removes to 50 per cent. alcohol.

LO BIANCO says killing with 0·5 per cent. chromic acid or with 0·1 per cent. chloral hydrate in sea water may be tried. *Phascolosoma* and *Phoronis* should be treated by the alcohol method, larvæ of *Sipunculus* with cocaine, § 21.

APEL (*Zeit. wiss. Zool.*, xlii, 1885, p. 461) puts *Priapulus* and *Halicryptus* into a vessel with sea water and heats on a water-bath to 40° C. ; or they may be thrown into boiling water, which paralyses them so that they can be quickly cut open and thrown into $\frac{1}{3}$ per cent. chromic acid or picro-sulphuric acid.

886. **Rotatoria.**—For quieting them for study in the living state, WEBER (*Arch. de Biol.*, viii, 4, 1888, p. 713) finds that 2 per cent. solution of hydrochlorate of cocaine gives the best results. Warm water gave him good results for large species, such as those of *Hydatina* and *Brachionus*.

HARDY (*Journ. Roy. Mic. Soc.*, 1889, p. 475) recommends thick syrup added drop by drop to the water. HUDSON (*ibid.*, p. 476) mentions weak solution of salicylic acid.

VOLK (*Jahrb. Hamburg. wiss. Anst.*, xviii, 1901, p. 164) quiets them in quince mucilage, 40 grm. of the seeds to 1 litre of water. *Cf.* § 917.

HIRSCHFELDER (*Zeit. wiss. Zool.*, xcvi, 1910, p. 211) studies them living in neutral red of 1 : 50,000.

See also §§ 23, 24 and 27. Methylene blue, § 339, may be found useful.

Permanent preparations may be made by the method of ROUSSELET (*Journ. Quekett Mic. Club*, v, March, 1895, p. 1) : The animals are got together in a watch glass and are narcotised by adding to the water at intervals a few drops of the following mixture:

Hydrochlorate of cocaine 2 per cent. solution	3	parts.
Methylated spirit	1	„
Water	6	„

As soon as the cilia have ceased to beat, or are seen to be on the point of ceasing to beat, they are fixed by adding a drop of liquid of Flemming or of ¼ per cent. osmic acid. After half a minute or less the animals are taken out with a pipette, and thoroughly washed by passing them through two or three watch glasses of distilled water. They are then definitely mounted in a mixture of formol 2½ parts, distilled water 37½ parts.

Zograf (*Comptes Rend.*, cxxiv, 1897, p. 245) narcotises as Rousselet, but without the spirit, fixes with osmic acid for two to four minutes, then replaces this by raw pyroligneous acid diluted with eight to ten volumes of water, and after five to ten minutes washes in several changes of water, and passes through successive alcohols into glycerin or balsam.

Lenssen (*La Cellule*, xiv, 1898, p. 428) for the embryology of *Hydatina*, kills with hot saturated sublimate, dehydrates, stains lightly, imbeds in paraffin and stains with hæmalum.

Hirschfelder (*op. cit. supra*) narcotises with cocaine, and fixes with Fol's picro-chromic acid.

Beauchamp (*Arch. Zool. Expér.*, iv, 1906, p. 29) finds 1 per cent. stovaïne better than cocaine for some forms. He (*ibid.*, x, 1909, p. 77) fixes for five to ten minutes in four parts of 1 per cent. osmic acid with one of 6 per cent. sublimate and five of 5 per cent. bichromate of potash, and one drop of acetic acid for each 2 c.c., and imbeds in celloidin, and then through chloroform in paraffin (three to ten minutes).

See also Tozer (*Journ. Roy. Micr. Soc.*, 1909, p. 24).

887. Acanthocephali.—Saefftigen (*Morph. Jahrb.*, x, 1884, p. 120) obtained the best results by killing gradually with 0·1 per cent. osmic acid; the animals placed in this contract during the first hours, but stretch out again and die fully extended. Similarly with 0·1 per cent. chromic acid; Echinorhynci live for days in it, but eventually die fully extended.

Hamann (*Jen. Zeit.*, xxv, 1890, p. 113) has succeeded with sublimate, and also with alcohol containing a little platinum chloride.

Kaiser (*Biblioth. Zool.*, H. vii, 1 Hälfte, 1891, p. 3) found that a saturated aqueous solution of cyanide of mercury, warmed to 45° to 50° C., and allowed to act for from fifteen to sixty minutes, and then washed out with 70 per cent. alcohol, was the best of all fixing media.

888. **Nematodes.**—The impermeable cuticle is a great obstacle to preparation. According to Looss (*Zool. Anz.*, 1885, p. 318) this difficulty may be overcome in the manner described in § 553.

For fixing, most recent authors recommend sublimate solutions; chromic solutions seem to have a tendency to make the worms brittle.

But, according to Zur Strassen (*Zeit. wiss. Zool.*, liv, p. 655), *Bradynema rigidum* ought to be fixed for at least twelve hours in mixture of Flemming.

Augstein (*Arch. Naturg.*, lx, 1894, p. 255) takes for *Strongylus filaria* Mayer's picro-nitric acid.

Vejdovsky (*Zeit. wiss. Zool.*, lvii, 1894, p. 645) advises for *Gordius* 0·5 per cent. chromic acid (twenty-four hours).

Lo Bianco employs for marine forms concentrated sublimate or picro-sulphuric acid.

Looss (*Zool. Anz.*, xxiv, 1901, p. 309) prefers hot (80° to 90° C.) alcohol of 70 per cent.

Glaue (*Zeit. wiss. Zool.*, xcv, 1910, p. 554) kills *Ascaris* in a hot mixture of 100 parts of saturated sublimate, 100 of alcohol, and 1 of acetic acid.

Staining is frequently difficult, and sometimes alcoholic carmine, § 234a, is the only thing that will give fair results.

Braun (see *Journ. Roy. Mic. Soc.*, 1885, p. 897) recommends that small unstained Nematodes be mounted in a mixture of 20 parts gelatin, 100 parts glycerin, 120 parts water, and 2 parts carbolic acid, which is melted at the moment of using. Canada balsam, curiously enough is said to sometimes make Nematodes opaque.

Demonstration of living Trichinæ.—Barnes (*Amer. Mon. mik. Journ.*, xiv, 1893, p. 104) digests trichinised muscle (of the size of a pea) in a mixture of 3 gr. of pepsin, 2 dr. of water, and 2 minims of hydrochloric acid, kept at body temperature for about three hours. The flesh and cysts being dissolved, the fluid is poured into a conical glass, and allowed to settle; the trichinæ are drawn off from the bottom with a pipette, got on to a slide with water and examined on a hot stage.

Graham (*Arch. mik. Anat.*, l, 1897, p. 216) isolates Trichinæ by macerating for one or two days in 2 per cent. acetic acid, staining with aceto-carmine, and teasing.

889. Nemertina.—My best results have always been obtained by fixing with cold saturated sublimate solution, acidified with acetic acid. The other usual fixing agents, such as the osmic and chromic mixtures, seem to act as irritants, and provoke such violent muscular contractions that the whole of the tissues are crushed out of shape by them.

Prof. DU PLESSIS has suggested to me fixing with hot (almost boiling) water. I have tried it and found the animals die in extension, without vomiting their proboscis. So also JOUBIN, *Bull. Mus. Hist. Nat.*, 1905, p. 326.

I have tried FOETTINGER's chloral hydrate method (§ 20). My specimens died fairly extended, but vomited their proboscides. According to LO BIANCO narcotisation with a solution of 0·1 to 0·2 per cent. in sea water for six to twelve hours is useful.

OESTERGREN (§ 18) recommends his ether water.

DENDY (see *Journ. Roy. Mic. Soc.*, 1893, p. 116) has succeeded with *Geonemertes* by exposing it for half a minute to the vapour of chloroform.

For staining fixed specimens *in toto* I have found that it is well-nigh necessary to employ *alcoholic* stains. Borax-carmine or Mayer's alcoholic carmine may be recommended; not so cochineal or hæmatoxylin stains, on account of the energy with which they are held by the mucin in the skin.

Sections by the paraffin method, after penetration with *oil of cedar* (chloroform will fail to penetrate sometimes after a lapse of weeks).

BÜRGER (*Fauna u. Flora Golf. Neapel*, xxii, 1895, p. 443) studies the nervous system, nephridia, skin, muscle and intestine by the *intra vitam* methylen-blue method. He *injects* the animals with 0·5 per cent. solution in distilled water, or 0·5 per cent. salt water, and allows them to lie for six to twelve hours or more in moist blotting paper.

See also MONTGOMERY (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 6); and BÖHMIG (*Zeit. wiss. Zool.*, lxiv, 1898, p, 484).

890. Cestodes.—As pointed out by VOGT and YUNG (*Traité d'Anat. Comp. Prat.*, p. 204), the observation of the living animal may be of service, especially in the study of the excretory system. And, as shown by PINTNER, Tæniæ may be preserved alive for several days in common water to which a little white of egg has been added.

TOWER (*Zool. Jahrb.*, xiii, 1899, p. 363) has kept *Moniezia expansa* alive for several days in a mixture of 100 c.c. of tap water, 10 gr. of white of egg, 2 of pepsin, 2 of sugar, and 5 of prepared beef ("Bovox"). Chloride of sodium, he says, should be avoided.

LÖNNBERG (*Centralb. Bakteriol.*, xi, 1892, p. 89; *Journ. Roy. Mic. Soc.*, 1892, p. 281) has kept *Triænophorus nodulosus* alive for a month in a slightly acid pepsin-peptone solution containing from 3 to 4 per cent. of nutritive matter and less than 1 per cent. of NaCl.

For the nervous system, TOWER (*Zool. Anz.*, xix, 1896, p. 323) fixes in a picro-platin-osmic mixture (stronger than that of O. vom RATH, § 101) for ten hours, then treats for several hours with crude pyroligneous acid, and lastly with alcohol, and imbeds in paraffin.

ZERNECKE (*Zool. Jahrb., Abth. Anat.*, ix, 1895, p. 92) kills *Ligula* in the osmio-bichromic mixture of GOLGI (4 : 1), impregnates as usual, makes sections in liver, and treats them by the hydroquinon process of KALLIUS. Besides the peripheral and central nervous system, muscle-fibres, parenchyma cells, and the excretory vascular system are impregnated.

He has also obtained good results by the methylen-blue method.

BLOCHMANN (*Biol. Centralb.*, xv, 1895, p. 14) recommends the bichromate and sublimate method of GOLGI.

See also KÖHLER, *Zeit. wiss. Zool.*, lvii, 1894, p. 386 (stretches Tæniæ round a glass plate or on cork, and fixes with 5 per cent. sublimate); LÜHE, *Centralb. Bakt.*, xxx, 1901, p. 166, and RANSOM, *U. S. Nation. Mus. Bull.*, lxix, 1909, p. 8.

891. Trematodes (FISCHER, *Zeit. wiss. Zool.*, 1884, p. 1).—*Opisthotrema cochleare* may be mounted entire in balsam. For sectioning, he recommends a mass made by dissolving 15 parts of soap in 17·5 parts of 96 per cent. alcohol. The sections should be studied in glycerin.

LO BIANCO fixes Trematodes with hot saturated sublimate.

LOOSS (*Arch. mik. Anat.*, 1895, p. 7) takes for *Bilharzia* warm (50° to 60° C.) 1 per cent. sublimate in 70 per cent. alcohol.

Bettendorf (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 308) has had good results with the rapid Golgi method only on *Distoma hepaticum*, and prefers methylen blue.

Havet (*La Cellule*, xvii, 1900, p. 353) has also had results with the Golgi method on this form, and also with thionin, (after fixing with sublimate), which demonstrates tigroid substance.

Cercariæ.—Schwarze (*Zeit. wiss. Zool.*, xliii, 1886, p. 45) found that the only fixing agent that would preserve the histological detail of these forms was cold saturated sublimate solution warmed to 35°—40° C.

For an "indifferent" liquid, Hofmann (*Zool. Jahrb.*, xii, 1899, p. 176) takes 1 part of white of egg in 9 of normal salt solution.

892. Turbellaria.—Braun (*Zeit. wiss. Mik.*, iii, 1886, p. 398) gets entire animals (Rhabdocœla) on to a slide, lightly flattens out with a cover, and kills by running in a mixture of three parts of liquid of Lang with one of 1 per cent. osmic acid solution. (Böhmig [*ibid.*], commenting on this, says that for some of the tissues, such as muscle and body parenchyma, nitric acid and picro-sulphuric acid are very useful.) Sections may be made by the paraffin method.

Delage (*Arch. de Zool. exp.*, iv, 2, 1886) recommends fixation (of Rhabdocœla Acœla) by an osmium-carmine mixture, for which see *loc. cit.*, or by concentrated solution of sulphate of iron. Liquid of Lang was not successful.

For staining, he recommends either the osmium-carmine or impregnation with gold ($\frac{1}{3}$ formic acid, two minutes; 1 per cent. gold chloride, ten minutes; 2 per cent. formic acid, two or three days in the dark.)

Böhmig (*Zeit. wiss. Mik.*, iii, 1886, p. 239) has obtained instructive images with Plagiostomidæ fixed with sublimate and stained with the osmium-carmine.

Graff (*Turbellaria Acœla*, Leipzig, 1891; *Zeit. wiss. Mik.*, ix, 1892, p. 76) says that chromo-aceto-osmic acid, followed by hæmatoxylin, is good for the skin, but not for the Rhabdites, which in Acœla and Alloiocœla seem to be destroyed by swelling. The same method is also good for the parenchyma of *Amphichœrus cinereus*, *Convoluta paradoxa* and *C. sordida.* Sublimate is good for *Convoluta Roscoffensis.* The nervous system may be investigated by the methods of Delage.

For Dendrocœla sublimate solutions, sometimes hot, appear indicated for fixing; see the mixture of Lang, § 64, also Chichkoff (*Arch. de. Biol.*, xii, 1892, p. 438).

Arnold (*Arch. Zellforsch.*, iii, 1909, p. 433) kills *Dendrocœlum* in extension (?) with strong liquid of Flemming.

Oestergren narcotises *Dendrocœlum* with his ether-water, § 18.

Jaenichen (*Zeit. wiss. Zool.*, lxii, 1896, p. 256) advises for *Planaria*, eyes epecially, picro-sulphuric acid for an hour or two; osmic acid is not good, and liquid of Müller macerates. He stains with borax-carmine, makes sections, and puts them for ten minutes into osmic acid, then for five minutes into pyroligneous acid, on the top of the stove. He macerates the visual rods in a mixture of one part common salt, one of acetic acid, and 100 of water. He bleaches the pigment of the eyes with peroxide of hydrogen.

Wilhelmi (*ibid.*, lxxx, 1906, p. 548) throws Triclads into almost boiling mixture of Zenker, and after 10 to 30 minutes removes to water for some hours, and then passes into iodine alcohol.

Echinodermata.

893. Holothurioidea.—These are difficult to fix on account of their contracting with such violence under the influence of irritating reagents as to expel their viscera through the oral or cloacal aperture.

Vogt and Yung (*Anat. Comp. Prat.*, p. 641) say that *Cucumaria Planci* (*C. doliolum,* Marenzeller) is free from this vice; but they recommend that it be killed with fresh water, or by slow intoxication, § 25.

Synapta may be allowed to die in a mixture of equal parts of sea water and ether or chloroform (S. Lo Bianco).

Oestergren (§ 18) puts *Synapta* into his ether water, but Dendrochirota first into magnesium sulphate of 1 to 2 per cent., for some hours.

Gerould (*Bull. Mus. Harvard Coll.*, xxix, 1896, p. 125) paralyses *Caudina* with sulphate of magnesia, § 24, and fixes with liquid of Perényi (or sublimate for the ovaries).

Holothurids, Dr. Weber informs me, are admirably preserved in formaldehyde; a weak solution is sufficient.

For the staining of muscles with methylen blue, see Iwanzoff, *Arch. mik. Anat.*, xlix, 1897, p. 103; and for the study of calcareous plates, see Woodland, *Quart. Journ. Micr. Sci.*, xlix, 1906, p. 534 (fixation with osmic acid, staining with picro-carmine, followed by Lichtgrün).

894. Echinoidea.—I advise that they be killed by *injection* of some fixing liquid. For preservation, formaldehyde has proved *admirable* in all respects, and greatly superior to alcohol (Weber).

Lo Bianco kills by pouring over them (mouth upwards) a mixture of ten parts acetic acid and one of 1 per cent. chromic acid, and brings at once into weak alcohol. Or he makes two holes in the shell, lets the water run out and alcohol run in.

Sections of spines may be made by grinding, see § 177.

Spicula and the skeleton of pedicellariæ may be cleaned by *eau de Javelle*, see Döderlein (*Wiss. Ergeb. Tiefsee-Exped.*, v, 1906, p. 67).

895. Asteroidea.—Hamann (*Beitr. Hist. Echinodermen*, ii, 1885, p. 2) *injects* the living animal with a fixing liquid through the tip of a ray. The ambulacral feet and the branchiæ are soon distended by the fluid, and the animal is then thrown into a quantity of the same reagent.

In order to study *the eyes*, with the pigment preserved *in situ*, they should be removed by dissection, should be hardened in a mixture of equal parts of 1 per cent. osmic acid and 1 per cent. acetic acid, and sectioned in a glycerin gum mass, or some other mass that does not necessitate treatment with alcohol (which dissolves out the pigment, leaving the pigmented cells perfectly hyaline). For maceration use one-third alcohol, the aceto-osmic mixture failing to preserve the rods of the pigmented cells.

Formaldehyde is *not* to be recommended for the preservation of Asteroidea (Weber).

See also Lo Bianco, *op. cit.* (he kills *Brisinga* with absolute alcohol), also §§ 17, 20.

896. Ophiuridea should in general be killed in *fresh water* if it be desired to avoid rupture of the rays (De Castellarnau, *La Est. Zool. du Napoles*, p. 135).

Lo Bianco kills small forms with weak alcohol, *Ophiopsila* with absolute alcohol, and *Ophiomyxa* with 0·5 per cent. chromic acid.

Russo (*Richerche Lab. Anat. Roma*, iv, 1895, p. 157) fixes *Ophiothrix* for an hour or two in 0·5 per cent. osmic acid and then decalcifies in solution of Müller for six to ten days. Or he fixes for three minutes in a mixture of two parts concentrated sublimate solution, one part 70 per cent. alcohol, and one part acetic acid (sp. gr. 1·06), and decalcifies in Müller or in 70 per cent. alcohol with 10 per cent. of acetic acid. He stains with paracarmine.

897. **Crinoidea.**—Lo Bianco (*loc. cit.*, p. 458) fixes *Antedon rosacea* with 70 per cent. alcohol, *A. phalangium* with 90 per cent.

898. **Larvæ of Echinodermata** (from instructions written down for me by Dr. Barrois).—For the study of the metamorphoses of the Echinoidea and Ophiuridea it is necessary to obtain preparations that show the *calcareous skeleton preserved* intact (a point of considerable importance, since this skeleton frequently affords landmarks of the greatest value), and that give clear views of the region of formation of the young Echinoderm (which is generally opaque in the living larva). They should also possess sufficient stiffness to allow of the larva being turned about in any desired way, and placed in any position under the microscope.

Pluteus larvæ should be fixed in a cold saturated solution of corrosive sublimate, for not more than two or three minutes, then washed with water, and brought into dilute Mayer's cochineal (§ 235). This should be so dilute as to possess a barely perceptible tinge of colour. They should remain in it for from twelve to twenty-four hours, being carefully watched the while, and removed from it at the right moment and mounted in balsam, or, which is frequently better, in oil of cloves or cedar-wood.

Auricularia and Bipinnaria.—As above, but the earlier stages of the metamorphosis of Auricularia are better studied by fixing with osmic acid, staining with Beale's carmine, and mounting in glycerin.

Larvæ of Comatula are best fixed with liquid of Lang, and stained with *dilute* borax-cramine. It is important (for preparations that are not destined to be sectioned) to use only *dilute* borax-carmine, as the strong solution produces an over-stain that cannot easily be reduced.

Narcotisation by chloral hydrate before fixing is useful, especially for the study of *Pentacrinus* larvæ and of the young *Synaptæ* formed from Auricularia. Without this precaution you generally get preparations of larvæ either shut up (*Pentacrinus*), or entirely deformed by contraction (young *Synaptæ*).

See also MacBride on the development of *Amphiura squamata*, *Quart. Journ. Micr. Sci.*, xxxiv, 1892, p. 131 (osmic acid followed by liquid of Müller and alcohol; decalcification with nitric acid in alcohol; staining with Mayer's paracarmine or hæmalum); and Seeliger on the development of *Antedon*, *Zool. Jahrb.*, *Abth. Anat.*, vi, 1892, p. 161.

Macbride (*Quart. Journ. Micr. Sci.*, xxxviii, 1896, p. 340) fixes larvæ of *Asterina* in osmic acid, brings into liquid of Müller for twelve to fourteen hours, imbeds in celloidin followed by paraffin (see § 171). and stains sections with carmalum or Delafield's hæmatoxylin, best after a foregoing stain of twenty-four hours in borax carmine.

Mayer (*Grundzüge*, Lee and Mayer, 1910, p. 486) arranges a number of fixed and stained *Plutei* on a sheet of gelatin foil gummed to a slide with euparal, dehydrates by adding alcohol by drops, and adds euparal and a cover. See also Woodland, *Quart. Journ. Micr. Sci.*, xlix, 1905, p. 307.

Cœlenterata.

899. Thread-cells.—Iwanzoff (*Bull. Soc. Nat. Moscou*, x, 1896, p. 97) advises for the Nematocysts of Actiniæ maceration by the Hertwig's method, § 534, or better, fixation for two to five minutes with vapour of osmium followed by a short washing with sea water or distilled water.

For Medusæ he also advises the Hertwig's method, § 534. or treatment with a solution containing methyl green and gentian violet with a little osmic acid.

900. Little (*Journ. App. Mic.*, vi, 1903, p. 2116; *Journ. Roy. Mic. Soc.*, 1903, p. 237) kills *Hydra* in hot saturated sublimate in 70 per cent. alcohol, washes with alcohol, stains for five minutes in strong solution of methylen blue, dehydrates rapidly, clears with cedar or bergamot oil, and mounts in balsam. Nematocytes blue, the rest unstained.

901. **Actinida.**—For *narcotisation* methods see §§ 15 to 26.

902. *Fixation.*—In *Le Attinie, Fauna u. Flora d. Golfes v. Neapel*, ANDRES says that hot corrosive sublimate often gives good results. In the case of the larger forms the solution should be injected into the gastric cavity.

Freezing sometimes gives good results. A vessel containing Actiniæ is put into a recipient containing an ice-and-salt freezing mixture and surrounded by cotton-wool. After freezing, the block of ice containing the animals is thawed in alcohol or some other fixing liquid.

DUERDEN (*Journ. Inst. Jamaica*, ii, 1898, p. 449) narcotises with magnesium sulphate, § 24, and fixes with formol of 3 to 5 per cent.

903. *Maceration.*—For the HERTWIGS' method (*Jen. Zeit.*, 1879, p. 457) see § 534. The tissues should be left to macerate in the acetic acid for at least a day, and may then be teased in glycerin.

LIST (*Zeit. wiss. Mik.*, iv, 1887, p. 211) treats tentacles of *Anthea cereus* and *Sagartia parasitica* for ten minutes with a mixture of 100 c.c. of sea water with 30 c.c. of Flemming's strong liquid, then washes out for two or three hours in 0·2 per cent. acetic acid, and teases in dilute glycerin. Picro-carmine may be used for staining.

904. *Nervous system.*—This group is generally held to be refractory to the Golgi impregnation. HAVET, however (*La Cellule*, xviii, 1901, p. 388), has obtained good results by the rapid method on young specimens of *Metridium dianthus*. Besides nerve-cells, there are impregnated neuro-muscular cells, gland-cells, and nematocytes. Leave for 5 to 8 days in the osmic micture. He has also had good results by the *intra vitam* methylen blue method (this is also good for nematocytes). So also has GROSELJ (*Arb. Zool. Inst. Univ. Wien*, xvii, 1909, p. 269), adding the dye to the water with the animals till it gives a steel-blue tint.

905. Zoantharia with Calcareous Skeletons are difficult to deal with on account of the great contractility of the polyps. Sublimate solution, which ought very often to be taken boiling, sometimes gives good results.

See also Lo Bianco, *loc. cit.*, p. 416.

Sections.—See §§ 177 and 178, for undecalcified specimens.

906. The Alcyonaria have also extremely contractile polyps. In a former edition I suggested for their fixation either hot sublimate solution or glacial acetic acid (§ 84). S. Lo Bianco has since recommended essentially similar processes. Garbini (*Manuale*, p. 151) drenches them with ether, and brings into strong alcohol.

Wilson (*Mitth. Zool. Stat. Neapel*, 1884, p. 3) kills Alcyonaria with a mixture of one part of strong acetic acid and two parts of concentrated solution of corrosive sublimate, the animals being removed as soon as dead and hardened for two or three hours in concentrated sublimate solution.

907. Zoantharia and Alcyonaria.—Braun (*Zool. Anz.*, 1886, p. 458) inundates *Alcyonium palmatum*, *Sympodium coralloides*, *Gorgonia verrucosa*, *Caryophyllia cyathus*, and *Palythoa axinellæ* with a mixture of 20 to 25 c.c. of concentrated solution of sublimate in sea water with four to five drops of 1 per cent. osmic acid, and after five minutes passes into successive alcohols.

(This method also gives good results with *Hydra* and some Bryozoa and Rotifers.)

See also § 14.

Bujor (*Arch. Zool. expér.*, ix, 1901, p. 50) kills *Veretillum* in sea water containing 10 per cent. each of formol and ether, and after a minute passes into 2 per cent. solution of formol in sea water.

908. Hydroidea, Polypoid Forms.—For suitable *narcotisation* methods see §§ 15 *et seq.*

For killing by *heat* see § 13.

Fixation.—In general the polyps may be very well killed in saturated sublimate solution, in which they should be plunged for an instant merely, and be brought into alcohol.

The solution should be employed cold in general for Gymnoblastea, hot for most Calyptoblastea.

Ether attentively administered gives good results with Campanularidæ. *Hydra* is very easily killed by a drop of osmic acid on a slide.

For the *methylen-blue intra vitam* method, see Chapter XVI; also Hadži, *Arb. Zool. Inst. Wien*, xvii, 1909, p. 225.

909. Medusæ: Fixation.—For narcotisation see § 17.

Trachymedusæ and Acalephæ may be fixed in the usual way in chromic or osmic mixtures. Osmic acid may be added to the sea water containing the animals, which should be removed to spring water as soon as they begin to turn brown.

910. Bigelow (*Mem. Boston Soc. nat. Hist.*, v, 1900, p. 193) fixes the scyphistomes of *Cassiopeia* in Lo Bianco's mixture of 10 parts of 10 per cent. solution of cupric sulphate with 1 of saturated sublimate, and hardens them in 5 per cent. bichromate of potash.

911. Medusæ: Sections.—Paraffin and collodion are certainly not satisfactory as all-round methods for these watery organisms. The Hertwigs (*Nervensystem der Medusen*, 1878, p. 5) imbedded in liver with the aid of glycerin gum, and hardened the objects and the mass in alcohol.

See also Joliet's glycerin-gum method, and the gelatin methods in Chapter VIII.

912. Medusæ: Maceration.—See, especially for the study of the nervous system, § 534. Doubtless in many cases the pyrogallic acid reaction, § 374, would give enhanced differentiation.

913. Siphonophora.—For the cupric sulphate method of Bedot (*Arch. Sci. phys. et nat.*, xxi, 1889, p. 556), which is admirable for the preparation of museum specimens, but not necessary for histological work, as well as for those of Lo Bianco (*op. cit.*, p. 454), Friedländer (*Biol. Centralb.*, x, 1890, p. 483), and Davidoff (*Anat. Anz.*, xi, 1896, p. 505) see *previous editions*. Lo Bianco fixes most forms with the mixture given § 910.

For preserving, according to Weber, formaldehyde is better than alcohol. Davidoff (*loc. cit.*) fixes in it.

914. Ctenophora: Fixation.—The small forms are very easily prepared by means of osmic acid. For the large forms see Lo Bianco, *loc. cit.*, p. 457. He uses his copper sulphate mixture, § 910.

Samassa makes sections by the double-imbedding method, see *Arch. mik. Anat.*, xl, 1892, p. 157.

Porifera.

915. Spongiæ: Fixation.—The smaller forms can be fairly well fixed by the usual reagents, osmic acid being one of the best. For the larger forms absolute alcohol is apparently the best. If any watery fluid be preferred, care should at all events be taken to get the sponges into strong alcohol as soon as possible after fixation, on account of the rapidity with which maceration sets in in watery fluids. Fiedler (*Zeit. wiss. Zool.*, xlvii, 1888, p. 87) has been using (for *Spongilla*), besides absolute alcohol, an alcoholic sublimate solution and the liquids of Kleinenberg and Flemming.

Staining.—To avoid maceration, I hold that alcoholic stains should be alone employed, and I recommend Mayer's tincture of cochineal, § 235. Von Lendenfeld (*Zeit. wiss. Mik.*, xi, 1894, p. 22) uses aqueous solutions of Congo red and anilin blue for the coloration of collar-cells.

Minchin (*Quart. Journ. Mic. Sci.*, xl, 1898, p. 569) stains spicula sheaths with Freeborn's picro-nigrosin, § 681.

Rousseau (*Ann. Soc. Belg. Mic.*, xxiv, 1899, p. 51) stains in nigrosin, picro-nigrosin, or indulin, or Mayer's picro-magnesia carmine.

For *intra-vitam* staining, see Loisel, § 208 *ante*, p. 138.

For silvering, see § 356.

Sectioning. — Calcareous sponges may be decalcified in alcohol, acidified with hydrochloric or nitric acid, and then imbedded in the usual way. Siliceous sponges may be desilicified, § 574.

For Rousseau's methods, see § 574. Vosmaer and Pekelharing decalcify with a solution of picric acid in absolute alcohol (see *Zeit. wiss. Mik.*, xv, 1899, p. 462).

See also Johnstone-Lavis and Vosmaer, § 179.

Preparation of Hard Parts.—Siliceous spicules are easily cleaned by treating them on a slide with hot concentrated nitric or hydrochloric acid, or solution of potash or soda. The acids mentioned are very efficient, but may attack the silex of some delicate spicules. Potash solution is, therefore, frequently to be preferred, notwithstanding that, in my experience, it does not give such clean preparations.

According to NOLL, *eau de Javelle* is preferable to any of these reagents, see § 552.

Embryos and Larvæ.—MAAS (*Zool. Jahrb., Abth. Morph.*, vii, 1894, p. 334) fixes larvæ in liquid of Flemming or Hermann, one to three minutes, and stains with borax-carmine, or with gentian violet and Orange G (Flemming). He also (*Zeit. wiss. Zool.*, lxvii, 1900, p. 218) fixes young Sycones in absolute alcohol and stains with ammonia carmine (spicules *in situ*).

DELAGE (*Arch. Zool. Expér.*, x, 1892, p. 421) fixes larvæ of *Spongilla* that have settled down on cover-glasses for three minutes in absolute alcohol, stains in alcoholic carmine, § 234A, and brings through alcohol into oil of bergamot, then either mounts direct in balsam, or detaches the larvæ from the cover and imbeds in paraffin (three minutes).

Protozoa.

916. Introductory.—The reagents and methods of cytology are in great part applicable to this group. One of the most generally useful of these reagents will be found in the acid solution of *methyl green;* it is the reagent that allows of the readiest and best demonstration of the presence and form of the nucleus and nucleolus (BALBIANI et HENNEGUY, *Compt. rend. Soc. de Biol.*, 1881, p. 131).

Weak solutions of alum, potash, and borax serve to demonstrate the striations of the cuticle, and the insertions of the *cilia* of Infusoria.

SCHUBERG (*Arch. Protistenk.*, vi, 1905, p. 63) stains cilia by the GOLGI impregnation (will bear a cover); or by LOEFFLER'S stain for flagella (fix with vapour of osmium, the rest under a cover).

For the *mitochondria* of Protozoa, see FAURÉ-FREMIET, *Arch. d'Anat. micr.*, xi, 1910, p. 457 (*intra-vitam* stains for them, Dahlia in salt solution or PICTET'S liquid being the best).

917. Immobilisation.—See the narcotisation methods §§ 20 to 25.

According to SCHÜRMAYER (*Jen. Zeit.*, xxiv, 1890, p. 402), nitrate of strychnin, of 0·01 per cent. or less, gives good results with some forms, amongst which are *Stentor* and *Carchesium*. Antipyrin (0·1 per cent.), or cocaine of 0·01 per cent., seems only to have given good results as regards the extension of the stalk in stalked forms.

EISMOND (*Zool. Anz.*, xiii, 1890, p. 723) slows the movements of small organisms (small worms and Crustacea as well as Ciliata) by means of a drop of thick aqueous solution of cherry-tree gum added to the water containing them (gum arabic and the like, it is stated, will not do). The objects remain fixed in their places, with cilia actively moving, and all vital processes retaining their full activity.

CERTES (*Bull. Soc. Zool. France*, xvi, 1891, p. 93) has found that an *intra vitam* stain may be obtained by adding methyl blue or "violet dahlia, No. 170" to the gum solution.

JENSEN (after STAHL; see *Biol. Centralbl.*, xii, 1892, p. 558) makes a solution of 3 grammes of gelatin in 100 c.c. of ordinary water by the aid of heat. This makes a jelly at the normal temperature. It is slightly warmed, and a drop of it is mixed in a watch glass with a drop of water containing the organisms.

See also VOLK, *ante*, § 886; STATKEWITSCH, *Arch. Protistenk.*, v, 1904, p. 17; LYON, *Amer. Journ. Phys*, xiv, 1905, p. 427 (*neutralised* gum).

918. Staining *intra vitam*.—See hereon BRANDT (*Verh. physiol. Ges. Berlin*, 1878); CERTES (*Bull. Soc. Zool.*, 25 janv., 1881); and HENNEGUY (*Soc. Philom.*, 12 fév., 1881). See also § 208.

BRANDT recommends a 1 : 3000 solution of Bismarck brown; also (*Biol. Centralb.*, i, 1881, p. 202) "a dilute solution of hæmatoxylin."

CERTES (*op. cit.*, pp. 21, 226, 264, and *Zool. Anz.*, iv, 1881, pp. 208, 287) found that living Infusoria stain in weak solutions of cyanin, Bismarck brown, dahlia, violet 5 B, chrysoidin, nigrosin, methylen blue, malachite green, iodine green, and other tar colours, and hæmatoxylin. The solutions should be made with the liquid that constitutes the natural habitat of the organisms. They should be very weak, that is of strengths varying between 1 : 10,000 and 1 : 100,000. For cyanin, 1 : 500,000 is strong enough.

As to the staining of the *Nucleus*, see PRZESMYCKI, *Biol. Centralb.*, vii, 1897, p. 321; and as to that of the *Granula*, the same author, *Zeit. wiss. Mik.*, xiii, 1896, p. 478. Also LOISEL, § 208,

Examination in a coloured medium in which the organisms do not stain, but show up on a coloured background is sometimes helpful. CERTES (*Bull. Soc. Zool. de France*, xiii, 1888, p. 230) recommends solution of anilin black—Infusoria will live in it for weeks; FABRE-DOMERGUE (*Ann. de Microgr.*, ii, 1889, p. 545) concentrated solution of diphenylamin blue.

919. Demonstration of Cilia (WADDINGTON, *Journ. Roy. Mic. Soc.*, 1883, p. 185).—A drop of solution of tannin, or a trace of alcoholic solution of sulphurous acid, added to the water containing the living organisms.

920. Fixing and Preserving.—For killing by *heat* see § 13.

PFITZNER (*Morph. Jahrb.*, xi, 1885, p. 454) used concentrated solution of picric acid *run in under the cover.*

ENTZ (*Zool. Anz.*, iv, 1881, p. 575) adds liquid of Kleinenberg to the water containing the organisms *in a watch glass.*

KORSCHELT (*ibid.*, v, 1882, p. 217) employs in the same way 1 per cent. osmic acid, or, for Amœbæ, 2 per cent. chromic acid.

LANSBERG (*ibid.*, p. 336) advises the same reagents, but recommends *bringing the organisms into the fixing liquid with a pipette.*

For fixation with iodine (KENT) or iodine vapour (OVERTON), see § 83.

For sulphurous acid, § 62.

CATTANEO (*Bollettino Scientifico*, iii and iv; *Journ. Roy. Mic. Soc.*, 1885, p. 538) fixes for a few minutes with $\frac{1}{3}$ per cent. solution of chloride of palladium.

BRASS (*Zeit. wiss. Mik.*, 1884, p. 39) employs a mixture of 1 part each of chromic acid, platinum chloride, and acetic acid with 400 to 1000 parts of water.

CERTES (*Comptes rend.*, lxxxviii, 1879, p. 433) fixes with

2 per cent. osmic acid, or its vapours (10 to 30 minutes). For details see *previous editions*.

Longhi (*Bull. Mus. Zool. Univ. Genova,* 1892, No. 4) kills in 10 c.c. of 1 per cent. sulphate of eserin with 1 drop of 1 per cent. sublimate.

Scala (*Rev. Mus. La Plata,* xv, 1908, p. 57) fixes for 5 or 10 minutes in a mixture of 2 mg. of atropin, 10 drops of formol, 10 grm. of glycerin and 50 c.c. of water.

See also Puschkarew, *Zeit. wiss. Mik.,* xxviii, 1911, p. 145 (agar process for fixing and staining Amœbæ).

Fol (*Lehrb.,* p. 102) fixes delicate marine Infusoria (*Tintinnodea*) with the perchloride of iron solution (§ 80), added to the water containing them, and stains with gallic acid, § 375.

Lo Bianco (*loc. cit.,* p. 444) fixes Gregarinæ with picro-sulphuric acid (one hour), Vorticellæ with hot sublimate, Acinetæ with sublimate in sea water, or with osmic acid, Thalassicola with 0·5 per cent. chromic acid (one hour), Acanthometræ and Aulacanthæ with 50 per cent. alcohol or with concentrated sublimate, or by adding a little osmic acid to the water. For Sphærozoa he proceeds as Brandt, § 922.

Zograf fixes Rhizopoda and Infusoria as Rotatoria, § 886, but without narcotisation.

See also Fabre-Domergue, *Ann. de Microgr.,* ii, 1889, p. 545, and 1890, p. 50; Schewiakoff, *Biblioth. Zool.,* v, 1889, p. 5; *Journ. Roy. Mic. Soc.,* 1889, pp. 832, 833; Zoja, *Boll. Sci. Pavia,* 1892; *Zeit. wiss. Mik.,* ix, 1893, p. 485; Lauterborn, *Zeit. wiss. Zool.,* lix, 1895, p. 170; Schaudinn, *ibid.,* p. 193; Balbiani, *Zool. Anz.,* xiii, 1890, p. 133; Karawaiew, *ibid.,* xviii, 1895, p. 286.

921. **Sections.**—The organisms should be strongly fixed, then dehydrated and cleared, and brought into melted paraffin in a small watch glass. After a few minutes therein they are brought on a cataract needle on to a small block of paraffin, and arranged there with a heated needle and sectioned. They may be stained after fixation, or the sections may be stained on the slide, §§ 186 or 187.

Entz (*Arch. Protistenk.,* xv, 1909, p. 98) brings the objects from clove oil into clove oil collodion of the consistency of honey, then brings them in this into a funnel made of paraffin, and when they have collected at the bottom of this puts it into chloroform, which dissolves the paraffin and hardens the collodion.

See also § 137, and PRZESMYCKI, *loc. cit.* § 918.

922. **Sphærozoa.**—BRANDT (*Fauna u. Flora Golf. Neapel*, xiii, 1885, p. 7) fixes with chromic acid of 0·5 per cent. to 1 per cent. (half an hour to an hour), or with a mixture of equal volumes of sea water and 70 per cent. alcohol with a little tincture of iodine for a quarter to half an hour, or with a 5 to 15 per cent. solution of sublimate in sea water.

KARAWAIEW (*Zool. Anz.*, xviii, 1895, p. 286) fixes *Aulacantha* for 24 hours in equal parts of strong liquid of Flemming and acetic acid, and hardens for several days in pure liquid of Flemming.

See also LO BIANCO, § 920.

923. **Sporozoa.**—WASIELEWSKI (*Sporozoenkunde*, Jena, 1896, p. 153) studies them living in their natural medium, or in normal salt solution, or in a medium composed of 20 parts white of egg, 200 of water, and 1 of common salt. He fixes Gregarinæ and Coccidia with osmic acid, sublimate, or picrosulphuric acid, and Myxosporidia with liquid of Flemming.

SCHAUDINN (*Zool. Jahrb., Abth. Anat.*, xiii, 1900, p. 197) fixes Coccidia with a mixture of 2 parts of saturated aqueous sublimate and 1 of absolute alcohol, with, if desired, a trace of acetic acid.

STEMPELL (*Arch. Protistenk.*, xvi, 1909, p. 389) fixes caterpillars infected with *Nosema* in two parts of saturated sublimate with one of alcohol and a little acetic acid, and stains sections for as much as four days in GIEMSA's mixture, rinses with alcohol and passes through xylol into balsam.

LÉGER (*ibid.*, iii, 1904, p. 311) fixes cysts for a minute in "acetic sublimate," puts for a minute into absolute alcohol, and stains as a smear with hæmalum or iron hæmatoxylin.

BRASIL (*Arch. Zool. Expér.*, 4, iv, 1905, p. 74) fixes them for twenty-four hours in a mixture of 1 grm. picric acid, 15 c.c. acetic acid, 60 c.c. formol and 150 c.c. alcohol of 80 per cent., and stains paraffin sections in iron hæmatoxylin followed by eosin and orange G., or Lichtgrün and picric acid.

924. **Hæmatozoa.**—GRASSI (*Att. Accad. Lincei*, iii, 1900, p. 357) demonstrates the Malaria-parasites in the intestine,

body-cavity and salivary glands of *Anopheles* by treating them with normal salt solution containing 2 per cent. of formol (pure formol produces swellings), or in a mixture of 1·5 grm. of salt and 250 c.c. of water with the white of an egg. He fixes with sublimate, makes paraffin sections, and stains with hæmalum or iron hæmatoxylin. He stains the Sporozoïtes by making cover-glass preparations which are allowed to dry, put for twenty-five minutes into absolute alcohol, and stained by the process of ROMANOWSKY, § 720.

For minute instructions for the application of this process to *sections*, see GIEMSA, *Deutsch. med. Wochenschr.*, xxxvi, No. 12, 1910; and SCHUBERG, *ibid.*, xxxv, No. 40, 1909 (*Zeit. wiss. Mik.*, xxvii, 1910, pp. 160, 161 and 513).

For *clinical methods*, see COLES, *The Diseases of the Blood*, London, J. & A. Churchill, 1905.

BRADFORD and PLIMMER (*Quart. Journ. Micr. Sci.*, xlv, 1902, p. 452) fix *Trypanosomes* in vapour of equal parts of acetic acid and 2 per cent. osmic acid, or with GULLAND'S formol and absolute alcohol, and stain with methylen blue and eosin, and mount in turpentine colophonium.

HINDLE (*Univ. Calif. Pub. Zool.*, vi, 1909, p. 129) makes smears on cover glasses coated with albumen, fixes for five minutes in liquid of Flemming, passes through water up to absolute alcohol, then for ten minutes into alcohol of 80 per cent. with a good proportion of iodine in potassic iodide, then into 30 per cent. alcohol, and stains with iron hæmatoxylin or safranin, then with polychrome methylen blue, and lastly with UNNA'S orange with tannin, and gets quickly through alcohol into xylol and balsam.

MINCHIN (*Quart. Journ. Micr. Sci.*, liii, 1909, p. 762) makes cover-glass smears, fixes them with vapours of osmic acid (with or without acetic acid), and mounts them dry, or in balsam after fixing in liquids and various stains, amongst these that of TWORT. Half-saturated solutions of neutral red and Lichtgrün are mixed, the precipitate dried and dissolved to about 0·1 per cent. in methyl alcohol with 5 per cent. of glycerin. Three parts of this are diluted with 1 of water, the smears stained for an hour, differentiated with UNNA'S glycerin-ether, and mounted in balsam. This stain works best after fixation with sublimate.

POLICARD (*C. R. Soc. Biol.*, lxviii, 1910, p. 505) stains

Trypanosomes *intra vitam* by adding a drop of concentrated solution of neutral red to the edge of a drop of blood spread between slide and cover.

925. Flagellata.—LAUTERBORN (*Zeit. wiss. Zool.*, lix, 1895, p. 170) fixes *Ceratium* for about ten minutes in liquid of Flemming, puts into alcohol for twenty-four hours, brings back into water, bleaches if necessary with hydrogen peroxide, and stains with picrocarmine or Delafield's hæmatoxylin. He also imbeds in paraffin, § 921, and stains sections with iron hæmatoxylin.

ZACHARIAS (*Zool. Anz.*, xxii, 1899, p. 72) fixes *Uroglena*, etc., with a mixture of 2 vols. saturated aqueous solution of boracic acid and 3 of saturated sublimate.

926. Stains for Flagella.—The ROMANOWSKY stain will give a red stain of the flagella of some forms.

The method of LÖFFLER (*Centralbl. Bakteriol.*, vi, 1889, p. 209; vii, 1890, p. 625; *Zeit. wiss. Mik.*, vi, 1889, p. 359; vii, 3, 1890, p. 368; *Journ. Roy. Mic. Soc.*, 1889, p. 711; 1890, p. 678) is as follows. To 10 c.c. of a 20 per cent. solution of tannin are added 5 c.c. of cold saturated solution of ferrous sulphate and 1 c.c. of (either aqueous or alcoholic) solution of fuchsin, methyl violet, or "Wollschwarz." Cover-glass preparations are made and fixed in a flame in the usual way, special care being taken not to over-heat. Whilst still warm the preparation is treated with mordant (*i. e.* the above-described mixture), and is heated for half a minute, until the liquid begins to vaporise, after which it is washed in distilled water and then in alcohol. It is then treated in a similar manner with the stain, which consists of a saturated solution of fuchsin in anilin water (p. 177), the solution being preferably neutralised to the point of precipitation by cautious addition of 0·1 per cent. soda solution.

See also LIEBETANZ, *Arch. Protistenk.*, xix, 1910, p. 23.

BUNGE (*Journ. Roy. Mic. Soc.*, 1894, p. 640; *Zeit. wiss. Mik.*, xiii, 1896, p. 96) makes the mordant by mixing three parts of the tannin solution with 1 of *liquor ferri sesquichlorati* diluted twentyfold with water, and lets the mixture ripen for some days exposed to the air, or (*Journ.*, 1895, pp. 129, 248) adds to it a few drops of hydrogen peroxide, until it becomes red-brown, when it is shaken up and filtered on to the cover-glass and allowed to act for a minute. The cover-glass is then mopped up and dried, and stained with carbol-gentian.

Koerner and Fischer (quoted from *Encycl. mik. Techn.* p. 514) make the mordant with 2 parts of tannin, 20 of water, 4 of ferrous sulphate solution of 1 : 2 strength, and 1 of saturated alcoholic solution of fuchsin. Warm, let it act for a minute, rinse and stain with anilin-water-fuchsin, or carbol-fuchsin.

Similarly Ellis (*Centralb. Bakt.*, xxi, 1903, p. 241; *Journ. Roy. Mic. Soc.*, 1904, p. 249), but staining with Säureviolett, 1 part to 75 of alcohol and 75 of water.

Peppler (*Centralb. Bakt.*, xxix, 1901, p. 376; *Zeit. wiss. Mik.*, xviii, 1901, p. 222) makes the mordant with 20 parts of tannin in 80 of water, and 15 parts of 2·5 per cent. chromic acid added gradually. This mordant will keep for months.

Rossi (*Arch. per le Sc. med.*, xxiv, 1900, p. 297; *Zeit. wiss. Mik.*, xviii, 1901, p. 226) takes for the mordant a solution of 25 grms. of tannic acid in 100 of caustic potash of 0·1 per cent., which will keep indefinitely. The stain is Ziehl's carbol-fuchsin, § 289. Cover-glasses are prepared with a drop of culture, dried, and treated with 1 drop of the mordant and at the same time 4 to 5 of the stain, allowed to remain for 15 to 20 minutes, washed, and mounted. See also *Centralb. Bakt.*, xxxiii, 1903, p. 572 (*Zeit. wiss. Mik.*, xix, 1903, p. 517).

Gemelli (*Centralb.*, xxxiii, 1903, p. 316; *Zeit. wiss. Mik.*, xix, 1903, p. 516) mordants for 10 to 20 minutes in 0·025 per cent. permanganate of potash, rinses and stains for 15 to 30 minutes in a mixture of 20 parts 0·75 per cent. aqueous solution of calcium chloride and 1 part of 1 per cent. neutral red solution.

A method of Pitfield is described by Kendall, *Journ. app. Mic.*, v, 1902, p. 1836 (*Journ. Roy. Mic. Soc.*, 1902, p. 502). The mordant consists of 10 parts of 10 per cent. tannin solution, 5 parts of saturated sublimate solution, 5 of saturated solution of alum, and 5 of carbol fuchsin. Mordant for a minute with heat, and stain with a mixture of 2 parts saturated aqueous solution of gentian violet with 10 of saturated solution of alum.

Van Ermengem (*Journ.*, 1894, p. 405) fixes for a few minutes with a mixture of 1 part 2 per cent. osmic acid, and 2 parts 10 to 25 per cent. solution of tannin, washes, treats with 0·25 to 0·5 per cent. solution of nitrate of silver, then for a few seconds with a mixture of 5 parts gallic acid, 3 of tannin, 10 of acetate of soda, and 350 of water, then puts back again into the silver for a short time, then washes and mounts.

See also Stephens, *ibid.*, 1898, p. 685, and Gordon, *ibid.*, 1899, p. 235, and the methods of Trenkmann (*Centralb.*, vi, 1889, p. 433; *Zeit. wiss. Mik.*, vii, 1890, p. 79); Brown (*Journ. Roy. Mic. Soc.*, 1893, p. 268); Julien (*ibid.*, 1894, p. 403); Sclavo (*Zeit. wiss. Mik.*, xiii, 1896, p. 96); Hessert (*ibid.*, p. 96); Muir (*Journ. Roy. Mic. Soc.*, 1899, p. 235); McCrorie (*ibid.*, 1897, p. 251; he stains for two minutes in a mixture of equal parts of concentrated solution of night-blue, 10 per cent. solution of alum, and 10 per cent. solution of tannic acid); Zettnow (*ibid.*, 1899, pp. 662, 664); Morton (*ibid.*, 1900, p. 131); Welcke (*ibid.*, p. 132); Levaditi, *C. R. Soc. Biol.*, lix, 1905, p. 326 (for *Spirochæte pallida*, Ramón's neurofibril stain); Meirowsky, *Münch. med. Wochenschr.*, lvii, 1910, No. 27; Kalb, *ibid.*, No. 26 (*Zeit. wiss. Mik.*, xxix, 1912, pp. 123, 124; both for *Spirochæte*).

APPENDIX.

927. Chemicals, Stains, and Apparatus.—Addresses from which it is recommended that these be obtained are given in § 11.

928. Cleaning Slides and Covers.—*New* ones should first be soaked in one of the following liquids: strong sulphuric, hydrochloric or nitric acid, or *aqua regia*, or a mixture of an ounce each of sulphuric acid and bichromate of potash with from 8 to 12 ounces of water, then washed first with water and lastly with alcohol, and dried with a clean cloth.

For *used* ones, if a balsam mount, warm, push the cover into a vessel with xylol or other solvent of the mount, and put the slide into another vessel with the same, leave for a few days, and then put into strong alcohol. If this is not sufficient, treat as for new ones. Some persons boil in lysol, which I do not find efficacious.

For the final treatment, see p. 121.

929. Gum for Labels.—Labels stuck on glass often strip off. This may be avoided (Marpmann, *Zeit. Angew. Mik.*, ii, 1896, p. 151; *Journ. Roy. Mic. Soc.*, 1897, p. 84) by means of the following adhesive: 120 grammes of gum arabic are dissolved in a quarter of a litre of water, and 30 grammes of gum tragacanth in a similar quantity. After a few hours the tragacanth solution is shaken until it froths, and mixed with the gum arabic solution. Strain through linen and add 150 grammes of glycerin previously mixed with 2½ grammes of oil of thyme.

Peirce (*Journ. app. Mic.*, ii, 1899, p. 627; *Journ. Roy. Mic. Soc.*, 1900, p. 404) finds that if the end of the slide be painted with a thin solution of balsam, it may be written on with ink when dry, and the record preserved by a second coat painted over it.

For other receipts see *early editions.*

INDEX.

The numbers refer to the pages.

A.

Abbe, mounting medium, 244
Absolute alcohol, 59—61, 235
Acanthocephali, 462
Acetate of copper, 56
Acetate of lead, 381
Acetate of potash, for bluing hæmatein stains, 160; for mounting, 238; refractive index, 235
Acetate of silver, 216
Acetate of uranium, 57
Acetic acid, action in fixing mixtures, 22, 23—24, 45, 54; fixing with, 53; Lo Bianco's "concentrated," 54; due proportions in mixtures, 54; various mixtures, 55 *et seq.*; for decalcification, 280
Acetic acid carmine, 145
Acetic alcohol, 55, 381; ditto with sublimate, 55
Acetic bichromate, 42
Aceto carmine, 145
Acetone, for celloidin imbedding, 105; for dehydration, 4; for hardening, 63; for imbedding, 81; chloroform, 15; formol, 67; sublimate solution, 48
Achard and Reynaud, impregnation, 213
Achúcarro, neuroglia, 442
Acid alcohol, 62
"Acid" dyes, 130
Acid, free, test for, 191
Acid fuchsin, 183
Acid magenta, 183
Acid rubin, 183
Acidophilous elements, 132
Acidophilous mixture, 193
Acids, *see* Acetic, Chromic, Hydrochloric, Nitric, Osmic, etc.
Acids, Congo red as a test for, 191
Acridin red, 350
Actiniæ, 13, 14, 17, 274, 470, 471
Actinida, 470, 471
Actol, 429
Adamkiewics, myelin stain, 418
Addresses, for instruments and reagents, 10, 11
Adipose tissue, 351, 352
Adjective staining, 133
Agababow, elastic tissue, 348
Agassiz and Whitman, pelagic ova, 305
Aguerre and Krause, neuroglia, 440
Albumen, examination media, 237, 239; freezing method, 118; injection mass, 267; section-fixing process, 122, 123, 125; removal from ova, 289, 300
Alcohol, for dehydration, 3; for preservation, 4; for narcotisation, 14; for fixing, 59—62; for hardening, 61; for maceration, 271; removal of, 5; absolute, 61, 235; acetic, 55; one-third, 62; hydrochloric acid, 62; tests for water, 62; table for diluting, 60; ethylic, index of refraction, 235
Alcohol balsam, 246
Alcohol, methyl, for narcotisation, 15; refractive index, 235
Alcoholic carmines, 148—150

The numbers refer to the pages.

Alcoholic cochineal, 150
Alcoholic formol, 65
Alcyonaria, 472
Alcyonella, 15
Alcyonium, 13
Aldehyde, 428
Alexander, reconstruction, 291
Alferow, silver impregnation, 216
Alfieri, bleaching, 285
Alizarin, artificial, 197; for nerve-tissue, 385, 414; for neuroglia, 327; for mitochondria, 329; *intra vitam*, 138
Alkanna for staining, 353
Allen, gum and glycerine, 240
Allen, methylen blue, 205
Allerhand, myelin stain, 417
Alt, axis-cylinder stain, 385
Altmann, bleaching, 31; osmicated fat, 352; osmic and bichromic mixture, 37; nitric acid, 39; corrosion, 277; fixatives for nuclei, 323; bioblasts, 328
Alum, ammonia, solubility, 163
Alum, ferric, 157
Alum-carmine, 142, 143; ditto with picric acid, 145
Alum-hæmatoxylin stains, 159 *et seq.*; general characters, 159; bluing them, 159
Aluminium chloride carmine, 144
Alzheimer, neuroglia, 441
Amann, lactophenol, 241; high refractive medium, 243
Amber varnish, 252, 253
Ambronn and Held, polarisation, 417
Ammar, résine de, 246
Ammonia-alum, 163
Ammonia-carmine, 146; how to neutralise, 259, 260.
Ammonia, chromate of, 44
Ammonio-nitrate of silver, 216, 398
Ammonium molybdate impregnation, 227, 401, 402
Amphibia, embryology, 300—303
Amphibia, larvæ, 317
Amphioxus, 305
Amphipoda, embryology, 313
Amyl nitrite, 258
Amyloid matter, 171
Andeer, phloroglucin, 283
Andreew, sulphorhodamin, 138
Andres, nicotine narcotisation, 13; Actiniæ, 14, 471; imbedding squares, 79; Actinida, 471
Andrews, imbedding apparatus, 79; osmic acid, 29; blastoderm of *Aves*, 296
Anethol, 118
Anglade and Morel, neuroglia, 441
Angus and Co., 10
Anile, section cutting, 94
Anilin, for clearing, 4, 73, 111; for imbedding, 73; as a mordant, 177; refractive index, 235
Anilin blue, 195, 230
Anilin blue-black, 196, 384
Anilin dyes, generalities, 169 *et seq.*; and *see* Coal-tar colours.
Anilin green, 170
Anilin oil, *see* Anilin.
Anilin red, 181
Anilin violet, 172
Anilin water, 177
Aniseed oil, 118, 235
Anitschkow, freezing method, 117; serial sections, 125
Annelids, 458; narcotisation, 15, 18, 458, 459
Anodonta, 16
Antennæ, 456
Anthozoa, 470
Anthracen ink stain, 437
Apáthy, washing sublimate material, 46; alcoholic sublimate, 48; osmic sublimate, 224; paraffin imbedding, 81, 83; knife position, 90, 91; knife-holder, 93; section-cutting, 96; paraffin mass, 98; gelatin imbedding, 101; celloidin imbedding, 103, 104, 105, 106, 108, 109, 110; serial sections, water method, 121; methods for celloidin sections, 126, 127; hæmatein mix-

The numbers refer to the pages.

ture I A, 164; alcoholic hæmotoxylin stain, 166; methylen blue, 200, 203, 205, 206, 207; cement for glycerin mounts, 240, 252; maceration, 274; muscle of Vermes, 342; gum syrup, 208, gold chloride, 219, 220, 223, 224; neuro-fibrils, 371, 402; formol mixture, 67; picro-säurefuchsin, 189; Canada balsam, 245; killing Hirudinea, 460
Apel, Gephyrea, 461
Apparato reticolare of Golgi, 403, 404
Aqueous humour, 237
Araneida, embryology, 312
Arctiscoida, 458
Areolar tissue, 345
Argentamin, 397, 417
Argulus, fixation of, 455
Argutinsky, serial sections, 125
Argyroneta, ova, 313
Arion, embryology, 308
Arndt, bone, 355
Arnold G., fixing mixture, 51; orange method, 190; *Dendrocœlum*, 467
Arnold, J., neutral red, 191; maceration, 271; glycogen, 320; cell granules, 327; mitochondria, 330; kidney, 372
Arnstein, methylen blue, 204, 207; gold method, 334; papillæ foliatæ, 335
Aronson, nerve-stain, with gallein, 414
Arsenic acid, 283
Artefacts of fixation, 22, 43
Arthropoda, 454—458; embryology, 309—313
Artificial alizarin, 197, 327, 329, 385, 414
Artificial fecundation, 287
Artificial serum, 238
Artom, embryology of *Ascaris*, 316
Ascaris, fixation, 463; ova, 55, 56, 194, 315
Ascidians, 17, 448; gemmation, 306; test-cells, 306
Asphalt varnish, 251; injection, 268
Asphyxiation, 17
Assmann, blood, 364
Astacus, embryology, 313
Asteroidea, 468; larvæ, 469; eyes, 457
Athanasiu and Dragoiu, connective tissue, 346
Atheson, Annelids, 460
Athias, Golgi impregnation, 429
Aubert, cements, 249
Auburtin, serial sections, 126
Auerbach, staining nerve-cells, 385, 389; axis-cylinder-stain, 385
Augstein, *Strongylus*, 463
Aurantia, 193
Auricularia, 469
Aves, embryology, 295—298
Axis-cylinder, stains for, 418 *et seq.*; structure, 389—403
Azofuchsin, 385
Azosäureblau, 385
Azoschwarz, 196
Azoulay, osmic acid nerve-stain, 227, 416; Golgi's impregnation, 425
Azur I, azur II, 200, 365
Azur-eosin, 365

B.

Babcock, celloidin blocks, 110
Babes, safranin, 177, 178
Bakay, cartilage, 358
Baker, C., address, 10
Balbiani, methyl green and eosin, 193; embryological methods, 288, 312; Protozoa, 478
Balbiani et Henneguy, Protozoa, 475
Ballowitz, muscle of Cephalopoda, 342; electric organs, 339, 340; embryologcal methods, 299
Balsam, Canada, 235, 245, 246
Balsam, for imbedding, 116, 117; as a cement, 252
Balsam, Tolu, 235
Balsams, 244

The numbers refer to the pages.

Balzer and Unna, elastic tissue, 347
Barium bichromate, 41
Barnes, Trichinæ, 463
Barrois, larvæ of Echinoderms, 469
Bartel, neuroglia, 440
Baryta water, 272
"Basic" dyes, 130
Basophilous elements, 132
Bastian, gold method, 222
Bataillon, ova of *Ascaris*, 315
Bataillon and Koehler, methylen blue, 324
Baumgarten, bleu de Lyon, 195; carmine and bleu de Lyon, 231
Bayerl, decalcification, 232; cartilage, 357; stain, 230
Bayon, neurofibrils, 390
Beale, shellac varnish, 253; injections, 265; digestion, 276
Beard, ova of *Raja*, 305
Beauchamp, Rotifers, 462
Beck, J., cements, 249
Becker, microtome, 11
Bedot, Siphonophora, 473
Bee, brain of, 457
Beer, medullated nerve, 405
Behrens, G., embryology of Salmonidæ, 304
Behrens, W., refractive indices, 234; maceration, 272; cement, 249, 252; clove oil, 71; levulose, 241; biniodide of mercury, 243
Béla Haller, maceration, 275
Bellarminow, injection, 268
Bell, fat, 353
Bell's cement, 251
Benario, blood, 362
Benda, nitric acid fixation, 39; iron hæmatoxylin, 155, 156; copper hæmatoxylin, 167; Lichtgrün stain, 194; Säureviolett stain, 194; neuroglia stains, 440; crystal violet, 197; mitochondria stain, 329; centrosome stains, 326; alizarin, 327, 329; secretion granules, 327; rapid myelin stain, 413; retina, 144; picro-säurefuchsin, 189, 344; fatty acids, 353
Benecke, stain for fibrils, 346
Benedecenti, formol, 64
Beneden, van, sublimate solution, 45; acetic acid, 54; acetic alcohol, 55; malachite green, 194; embryology of rabbit, 293, 294; of *Tænia*, 314; of *Ascaris*, 316.
Benedikt and Knecht, dyeing, 129
Bengal rose, 192
Bengtsson, larvæ of Diptera, 310
Bensley, fixing mixture, 51; intestine, 370; phosphorus, 320
Benzin colophonium, 246
Benzoazurin, 181, 197, 357
Benzol, for clearing, 73; for imbedding, 81, 83
Benzopurpurin, 191
Benzoyl green, 194
Berg, imbedding, 86
Bergamot oil, for clearing, 71, 235; for imbedding, 81
Bergh, Annelids, 459
Bergonzini, plasma cells and Mastzellen, 351
Berkley, rapid nerve-stain, 413; Golgi impregnation, 428; liver, 371
Berlin blue impregnation, 403
Berlin blue injections, 262, 266
Bernard, maceration of mollusca, 453
Berner, fat, 353
Bernheim, gold method, 223
Best, glycogen, 320
Besta, silver stain, 397; intracellular network, 404; myelin stain, 414
Bethe, treatment of osmic material, 31; methylen blue, 208; neurofibrils, 401; stain for chitin, 456; telsons, 456
Bettendorf, *Distoma*, 466
Betz, nervous centres, 375, 379
Bevan Lewis, *see* Lewis.
Bianco, S. Lo, *see* Lo Bianco.

The numbers refer to the pages.

Bichloride of mercury, *see* Sublimate.
Bichromate of ammonia, 44, 376, 379, 380
Bichromate of barium, 41
Bichromate of calcium, 41, 44
Bichromate of copper, 41, 415
Bichromate of potash, generalities, 41; for hardening, 41; for fixing, 40, and hardening, 376, 378, 379, 380; for maceration, 273
Bichromate of silver impregnation, *see* Golgi.
Bichromate and osmic mixtures, 37, 38; other mixtures, 42—44
Bichromate material, action of light on, 41; bleaching, 42, 285
Bichromates, 41
Bickfalvi, digestion, 277
Biedermann, methylen blue, 204; nerve and muscle, 337, 338
Bielaszewics, iron hæmatoxylin, 158
Bielschowsky, neurofibrils, 346, 390, 398, 399
Bielschowsky and Bruehl, inner ear, 446
Bielschowsky and Plien, nerve-cells, 389
Bielschowsky and Wolff, neurofibrils, 398
Bigelow, scyphistomes, 473
Binet, bleaching, 30; ganglia of Hexapods, 457
Bing and Ellermann, medullated nerve, 67
Biniodide of mercury mounting medium, 243
Bioblasts of Altmann, 328
Biondi, staining mixture, 184; blood, 360
Bipinnaria, 469
Bismarck brown, *intra vitam*, 136, 138; progressive, 172; regressive, 181
Bitume de Judée, 251
Bizzozero, blood-plates, 367; glands, 368
Bizzozero and Torre, blood, 363
Bjeloussow, injection, 267
Blackley blue, 195
Bladder of frog, 342
Blattida, embryology, 311
Blauschwarz, 191
Bleaching, osmic material, 30; bichromate material 42; chromic material, 33; in general, 284—286; gold material, 225
Bles, fixative, 65; ova of Amphibia 302
Bleu alcool, 195
Bleu Borrel, 366
Bleu carmin, 196
Bleu de Lyon, 195, 230
Bleu de nuit, 195
Bleu lumière, 195, 230
Blochman, series sections, 124; ova of Amphibia, 300; Cestodes, 465; Brachiopoda, 449
Blood, 359—368
Blood-parasites, 479—481
Blue-black, 196, 384
Blum, formol, 64, 65; celloidin imbedding, 108
Bobretzky, ova of Lepidoptera, 310
Boccardi, gold method, 223; stain for nerve-cells, 388
Bödecker, decalcification, 279, 356
Boeck, neurofibrils, 400
Böhm gold method, 222
Böhm and Oppel, artificial serum; 237; egg of fowl, 298; bichromate, 41; ova of reptiles, 299
Böhmer, hæmatoxylin, 163
Böhmig, Turbellaria, 466; Nemertina, 464
Bolton, myelin stain, 412; Golgi's impregnation, 428
Bone, 280—283, 353—357, and *see* Decalcification.
Bonnet, embryology of dog, 294
Bonney, triple stain, 190
Bonvicini, hardening brain, 380
Borax carmine, 148
Borax methylen blue, 324
Bordage, double imbedding, 115

The numbers refer to the pages.

Bordeaux R., 190, 191
Borgert, paraffin imbedding, 79
Boring, ova of *Ascaris*, 316
Born, section-stretcher, 94; reconstruction, 290, 291; ova of *Rana*, 302
Borrel, fixing mixture, 39; picro-indigo-carmine, 230
Borrel's blue, 366
Bouin, picric formol, 65, 66; sublimate formol, 66; larvæ of *Rana*, 303; ova of Salmonidæ, 304
Boule, neurofibrils, 397
Bouma, cartilage, 356
Boveri, picro-acetic acid, 58; imbedding small objects, 289; ova of *Ascaris*, 316
Boyce and Herdmann, copper, 320
Brachiopoda, 449
Bradford and Plimmer, trypanosomes, 480
Brady, chloral hydrate, 238
Braem, statoblasts, 306
Brain, *see* Neurological methods.
Branca, formol mixture, 66
Brandt, glycerin jelly, 242; infusoria, 476; Sphærozoa, 479
Brasil, Sporozoa, 479
Brass, paraffin, 81, 99; Protozoa, 40, 477
Braun, mounting medium for Nematodes, 463; Turbellaria, 466; Zoantharia and Alcyonaria, 472
Braus, fixation by injection, 25; ova of *Selachia*, 305; liver, 370
Brazilin, 228
Breckner, double imbedding, 114
Breglia, nerve stain, 414
Bremer, methylen blue and eosin, 193; Cox's sublimate impregnation, 433
Bresslau, Mesostomidæ, 314
Brillantschwarz, 191
Brinkmann, paraffin imbedding, 85
Brissy, freezing, 117
Bristol, bleaching, 30; Hirudinea, 460
Brock, maceration, 273
Broman, reconstruction, 291
Brookover, myelin stain, 407; Golgi impregnation, 430
Brown cement, 250
Brown, flagella, 483
Brücke, Berlin blue, 262; digestion, 277
Bruel, embryology of Diptera, 310
Brühl, corrosion, 277
Brun, glucose medium, 240
Bruno, mucus glands, 368
Brunotti, gelatin imbedding, 100
Brunswick black, 251
Bryozoa, 16, 449, 472; statoblasts, 306
Buchner, accessory chromosome, 324
Budge, injections, 268
Buds of Ascidians, 306
Buehler, staining nerve-cells, 389
Bujor, *Veretillum*, 472
Bumpus, thyme oil, 72; celloidin sections, 112
Bunge, stain for flagella, 481
Burchardt, E., bichromates, 41, 376; paraffin, 99; fixing mixtures, 41; pyroligneous-acid-carmine, 145; pyroligneous-acid-hæmatoxylin, 164; methyl green, 170; serial sections, 121; neuroglia, 442
Burckhardt, brain of *Protopterus*, 382
Bürger, Nemertina, 464
Burzynski, gelatin imbedding, 101
Busch, osmic mixture, 32; nerve-stain, 416; eosin, 231; decalcification, 280; glycogen, 320
Busse, celloidin, 103, 104, 106, 108
Bütschli, acid hæmatoxylin, 163; iron hæmatoxylin, 159
Buzzi, eleidin, 333

C.

Cade, gastric glands, 370
Cajal, Ramón y, *see* Ramón y Cajal.
Cajeput oil, 72

The numbers refer to the pages.

Calberla, methyl green, 170; ditto and eosin, 193; indulin, 195; glycerin mixture, 242
Calcium chloride, 235, 238; bichromate, 41
Calleja, picro-indigo-carmine, 230
Calvet, Bryozoa, 449
Cambridge microtomes, 10, 11
Camsal, camsal balsam, 247
Canada balsam, index, 235; for imbedding, 116; for mounting, 245, 246; as a cement, 252
Canfield, iris, 342
Caoutchouc cement, 249, 251
Capitellidæ, 15, 459
Capsicum, extract, 353
Carazzi, peroxide of sodium, 285
Carbol pyronin, 183
Carbolic acid, index, 235; for clearing, 73, 111
Carbolic fuchsin, 181
Carbon sulphide, refraction, 235; for imbedding, 82
Carbon tetrachloride, 81, 82
Carbonic acid for narcotisation, 18
Carmalum, 144, 146; with indigo-carmine, 230
Carmine, generalities, 141, 142; analysis, 141; stains in general, 142; formulæ for stains, 142 *et seq.*; aqueous, 142; alcoholic, 148—150; combination stains, 229—231
Carmine blue, 196
Carmine solutions, to neutralise, 259, 260
Carminic acid, 141
Carnoy, cajeput oil, 72; acetic alcohol, 55; Congo red, 191; salt solution, 236; tannin solution, 239; cement, 253; micro-chemical reactions, 319
Carnoy and Lebrun, ova of Amphibia, 300; ditto of *Ascaris*, 315; micro-chemistry, 320, 321
Carpenter, freezing, 117; section grinding, 115; cements and varnishes, 249
Carrière and Bürger, ova of *Chalicodoma*, 611
Carter, injection, 261
Cartilage, 356—358
Cassia oil, 71, 235
Castellarnau, de, Ophiuridea, 468
Castle, ova of *Ciona*, 306
Castor oil, 235, 248
Catois, methylen blue impregnation, 435
Cattaneo, palladium chloride, 52; Protozoa, 477
Caullery, Ascidians, 448
Causard, injection of spiders, 458
Caustic soda or potash, *see* Soda and Potash.
Cavazzani, hæmatoxylin and Säurefuchsin, 232
Cavalié, electric organs, 339
Cedar-wood oil, index, 235; for clearing, 70; imbedding, 81, 82, 83; for preserving, 5; for mounting, 245; for dissecting in, 8
Cell division, *see* Cytological methods.
Celloidin imbedding, 102 *et seq.*; generalities, 102, 103; preliminary preparation, 103; celloidin bath, 104; imbedding, 105; orientation, 106; hardening, 106—108; preserving blocks, 108; fixing blocks to microtome, 109; cutting, 110; clearing and mounting, 110; the new method, 112; Gilson's rapid, 112; the dry cutting method, 113; celloidin and paraffin method, 114; injections, 268
Celloidinum inelasticum, 103
Cells, paper for mounting, 250
Cells, study of, *see* Cytological methods.
Celluloid injection mass, 269
Cement for celloidin blocks, 109
Cements and varnishes, 249 *et seq.*
Central corpuscles, centrosomes, etc., 158, 325

The numbers refer to the pages.

Central nervous system, *see* Nervous centres and Neurological methods.

Cephalopoda, 450; embryology, 307; eyes, 451

Cercariæ, 466

Cerebrum, cerebellum, *see* Neurological methods.

CERFONTAINE, *Amphioxus*, 305; *Ascaris*, 316; *Lumbricus*, 459

CERTES, Infusoria, 476, 477

CESARIS-DEMEL, blood, 363

Cestodes, 464; embryology, 314

Chætopoda, 458—460

Chalicodoma, ova, 611

CHAMPY, mitochondria, 328

CHEATLE, dehydration apparatus, 3

Chemicals, 11

CHENZINSKY, methylen blue and eosin, 193

CHICHKOFF, Turbellaria, 467

CHILD, ova of fishes, 304; removal of albumen, 289

CHILESOTTI, nerve-stain, 384

Chilopoda, 455

Chinablau, 196, 405

China blue, 196, 405

Chinolinblau, chinolin blue, 195

Chitin, 278, 455, 456

Chiton, eyes, 452; embryology, 309

Chloral hydrate, for narcotisation, 15; preservative solutions, 238, 240, 243; for maceration, 275

Chlorate of potash for maceration, 274

Chloreton, 15

Chloride of aluminium carmine, 144

Chloride of calcium, 235, 238

Chloride of copper fixative, 56

Chloride of gold, *see* Gold chloride.

Chloride of iridium, 52

Chloride of magnesium, narcotisation, 17

Chloride of manganese, 237

Chloride of osmium, 52

Chloride of palladium, for fixing, 52; for staining, 227, 418; for decalcifying, 280

Chloride of platinum, 51; mixtures, 38, 40, 52, 289, 322

Chloride of ruthenium, 227

Chloride of sodium, *see* Salt.

Chloride of vanadium stain, 435

Chloride of zinc, 53, 381, 436

Chlorine for bleaching, 284

Chloroform, for narcotisation, 14; for clearing, 73; for imbedding, 81, 82, 83, 84

Cholesterin, 333

Chondriokonts, 328

Chondriosomes, 328

Choroid, 285

Chromate, neutral, of ammonia, 44

Chromate of lead impregnation, 227

Chromate of silver, *see* GOLGI.

Chromates, 40

Chromatin, reactions, 319

Chromatin stains, defined, 130; the coal-tar, 169—181; cytological, 324

Chromatophily, 132, 135

Chrome hæmatoxylin, 166

Chromic acid, generalities, 32; fixing with, 32; washing out, 32; hardening with, 34; action of light, 33; mixtures, 38 *et seq.*, 40, 67; for maceration, 273; for decalcification, 280, 283

Chromidia, 328

Chromium fluoride, 408, 410

Chromium sulphate for hardening, 380

Chromo-acetic acid, 35

Chromo-aceto-osmic acid, 35—37; for decalcification, 283

Chromo-formic acid, 35

Chromo-formol, 67

Chromogen, 439

Chromo-nitric acid, 39; for bleaching, 286

Chromo osmic acid, 35

Chromo-picric acid, 59

Chromo-platinic mixture, 40

Chromo sublimate, 50

CIACCIO, C., sublimate mixture, 48; fat, 351

The numbers refer to the pages.

Ciaccio, G. V., gold method, 222; corpuscles of Golgi, 341; cornea, 336
Ciaglinski, myelin stain, 418
Ciechanowski, liver, 370
Cilia of Infusoria, 475, 477
Ciliated epithelium, 453
Cilimbaris, muscle-spindles, 338; elastic tissue, 348
Cinnamon oil, 71, 235
Ciona, 17, 306
Citrate of silver, 216
Cladocera, 313
Clarke's spirit-proof cement, 251
Clasmatocytes, 351
Claudius, vegetal dyes, 229
Cleaning slides and covers, 121, 484
Clearing, generalities, 5, 68 *et seq*; clearing agents, 70 *et seq.*; practice of, 69; choice of an agent, 70; celloidin sections, 110; paraffin sections, 98
Clove oil, for minute dissections, 8, 71; refractive index, 235; for clearing, 70; for imbedding, 81; for differentiating stains, 175
Coal-tar colours, 169 *et seq*; regressive staining with, 173; progressive ditto, 170; choice of, 169; chromatin stains, 170—181; plasma stains, 182
Cobb, differentiator, 3
Cocaine, narcotisation, 16
Coccidæ, 456
Coccidia, 479
Cochineal, generalities, 141; aqueous, Partsch's, 143; Czokor's, 143; Rabl's, 144; alcoholic, Mayer's, 150; iron, 146
Cochlea, 445—447
Coe, Miracidia, 315
Cœlenterata, 470—474
Cœrulein, 194
Cohnheim, gold method, 220
Cole, freezing method, 118
Coleoptera, embryology, 311
Coles, parolein, 248; malaria parasites, 480
Collagen, 344
Collargol, 400
Collin, *Criodrilus*, 459
Collin and Lucien, intracellular network, 404
Collinge, pelagic ova, 305
Collodion for fixing sections, 123, 124, 125—128
Collodion imbedding, 102 *et seq.*; and *see* Celloidin
Collodionisation of sections, 95, 383
Colloidal gold, 401
Colloxylin, 103
Colophonium, for imbedding, 116; for mounting, 246; index, 235; cement, 252
Colour-acids, 130
Colour-bases, 130
Colucci, balsam, 246
Comatula, larvæ, 469
Combination stains, 228 *et seq.*; carmine combinations, 229; hæmatein ditto, 231
Congelation imbedding methods, 117
Congo red, 191, 385, 418, 474;—Corinth, 191
Conklin, ova of *Crepidula*, 308
Connective tissues, 344 *et seq.*
Conser, Bryozoa, 449
Cooling paraffin, 87
Copal, section method, 115; varnish, 253
Copepoda, 454; embryology, 313
Copper, detection of, 320
Copper, sulphate, 43, 303, 473; chloride and acetate, 56; impregnation, 227, 437; nitrate, 57, 67
Copper formol, 67
Copper hæmatoxylin, 167
Corals, 472
Corallin, 181
Cori, narcotisation, 15; cocaine, 16; keeping osmic acid, 28; chromo-aceto-osmic acid, 36
Cornea, 273, 331, 335
Corning, Krohnthal's impregnation, 434; medullated nerve, 405

The numbers refer to the pages.

Corpuscles, tactile, 334; of Herbst and Grandry, 334; of Meissner, 335; of Golgi, 340; of Meissnor and Krause, 335

Corti and Ferrara, pyronin, 183

Corrosion, 268, 277, 278

Corrosive sublimate, *see* Sublimate.

Cotton blue, 196

Coupier's blue, 195

Cox, Nissl's stain, 387, 389; medullated nerve, 405; sublimate impregnation, 433; neurofibrils, 403

Creasote, index, 235; for clearing, 73, 111; imbedding, 81

Creighton, glycogen, 320

Crepidula, 308

Cresyl violet, 197, 389

Crinoidea, 469

Cristatella, 15

Crustacea, 454; embryology, 313

Crystal violet, 197

Crystalline, 336

Csokor, cement, 252; bone, 355; and *see* Czokor.

Ctenophora, 474

Cuccati, soda carmine, 146

Cupric sulphate for fixing, 43, 473; for staining, 227

Curarisation, 317

Curreri, toning bath, 431

Curschmann, amyloid matter, 171

Cyanin, 195

Cyanide of mercury, 381

Cybulsky, gold impregnations, 225

Cyclas, ova, 309

Cytological methods, 317 *et seq.*; living cells, 317; fresh cells, 318; microchemical reactions, 319, 320; fixing agents, 321; chromatin stains, 324; plasma stains, 325; granules, 327; centrosomes, 325; nucleoli, 324; for nerve tissue, 386

Czokor, cochineal, 143; turpentine cement, 252

D.

Daddi, fat, 352

Da Fano, neuroglia, 441

Dahlgren, sublimate, 51; double imbedding, 114

Dahlia, 180

Dakin, injection of Acephala, 452

Damar (dammar), gum, 235, 246

Dantschakoff, serial sections, 125

Davidoff, ova of *Distaplia*, 306 Siphonophora, 473

Davies, injection, 261

De-alcoholisation, 5, 68 *et seq.*; choice of an agent, 70

Decalcification, 279—283

Decapoda, 454; embryology, 313

Decker, section-stretcher, 94

Deecke, encephalon, 380, 383

Deflandre, fat and lecithin, 351

Déjerine, sections of brain, 383

De Groot, serial sections, 121; iron-carmalum, 146; picro-magnesia carmine, 148; alcoholic hæmalum, 165

Dehydration, 2—5

Dekhuysen, silver impregnation, 216, 217; bichromate mixture, 43; blood, 361, 367; salt solution, 237

Delafield, hæmatoxylin, 163

Delage, Turbellaria, 466; larvæ of sponges, 475

Delamare, connective-tissue stain 346

Della Rosa, injection, 267

Della Valle, ova of *Orchestia*, 313

Deltapurpurin, 191

Dendy, *Geonemertes*, 464

Denker, corrosion, 277

Denne, paraffin imbedding, 84; orientation, 87

Depigmentation, 284—286

De Quervain, nervous tissue, 374

Desilicification, 283

De Vecchi, celloidin, 105

De Witt, elastic tissue, 348

Dewitz, injection of molluscs, 452

Dextrin freezing mass, 118

The numbers refer to the pages.

Differentiation, optical, by fixation, 20, 22, 37
Differentiator, Cobb's, 3; Haswell's, 3; Cheatle's, 3; Schultze's, 4; Kolster's, 4
Diffusion apparatus, 3
Digestion, 276, 277, 320
Dimmer, serial sections, 124
Dingler, mitochondria, 330
Diomidoff, nervous tissue, 381
Diptera, embryology, 310
Disse, stain for dentine, 358
Dissections, minute, 6, 8
Dissociation, methods of, 270
Distaplia, 306
Distoma, Miracidia, 315; staining, 466
Döderlein, Echinoidea, 468
Dogiel, methylen blue, 200, 203, 205, 207, 209, 210; corpuscles of Herbst and Grandry, 334, 392; corpuscles of Krause, 335; corpuscles of Golgi, 341; olfactive organs, 335; iris, 342; retina, 444
Dolbey & Co., address, 11
Döllken, formol imbedding method, 118; soap imbedding, 100
Donacia, ova, 311
Donaggio, tin hæmatoxylin, 168; neurofibrils, 402
Donaldson, nervous centres, 381
Dostoiewsky, iris, 342
Double imbedding, 114
Double stains, *see* Stains, combined.
Doyère, Arctiscoida, 458
Drasch, gold impregnation, 220
Dreuw, eleidin, 333
Dreyer, nervous system of Nudibranchiata, 451
Driessen, glycogen, 320
Drost, epithelium of mollusca, 453
Druener, fixation by injection, 25; osmic sublimate, 50; Ehrlich-Biondi stain, 185
Duboscq, blood, 361; Golgi impregnation, 428
Dubreuil, connective tissue, 345
Duerck, elastic tissue, 348
Duerden, Actiniæ, 471
Duesberg, mitochondria, 328, 330
Dunham, celloidin sections, 111
Du Plessis, Nemertians, 464
Durig, Golgi's impregnation, 427
Duval, collodion imbedding, 102, 103; silver impregnation, 215, 217; carmine and anilin blue, 230; embryology of birds, 295, 297; hardening brain, 380; purpurin, 229
Dyes, basic, acid, and neutral, 130; vegetal, 229

E.

Ear, inner, 445
Eau de Javelle, 278, 285; Eau de Labarraque, 278, 285
Ebner, von, decalcification, 282
Echinodermata, 18, 467—470
Echtgrün, 405
Edinger, liquid of Erlicki, 43; bleaching, 33
Edington, blood, 362
Eggs, of fowl, 295 *et seq.*; and *see* Embryological methods.
Ehlers, fixative, 35
Ehrenbaum, section grinding, 116
Ehrlich, acid hæmatoxylin, 164; classification of dyes, 132; triacid mixture, 187; acidophilous mixture, 193; mixture C (eosinophilous), 193; indulin-aurantia-eosin, 193; neutral red, 191; methylen blue, 200, 203; Mastzellen, 350; plasma cells, 350; quinolein blue, 195; eosinophilous cells, 193; blood films, 359
Ehrlich and Lazarus, glycogen, 320; neutral dyes, 131
Ehrlich-Biondi-Heidenhain stain, 184, 344
Ehrmann and Jadassohn, plasma fibrils, 332
Eichler, labyrinth, 447
Eimer and Amend, address, 11

Eisath, neuroglia, 441
Eisen, iridium chloride, 52; osmium chloride, 52; Ehrlich-Biondi stain, 186; ruthenium red, 227; Brazilin, 228; gum Thus, 248
Eisenberg, fat, 353
Eisig, alcohol narcotisation, 15; Capitellidæ, 15, 459; chromoplatinic mixture, 40; maceration, 273
Eismond, quieting Infusoria, 476
Ekman, Brachiopoda, 449
Elasthæmatein, 348
Elastic tissue, 344, 346—348; of spleen, 371
Electric organs, 339
Electrification of paraffin, 97
Eleidin, 333
Ellis, flagella, 482
Elschnig, celloidin, 104
Embryological methods, 287 *et seq.*; generalities, 287—291; Amphibia, 300; Arthropoda, 309—313; Aves, 295—298; Bryozoa, 306; Echinodermata, 469; Mammalia, 291—295; Mollusca, 307—309; Pisces, 303—306; Porifera, 475; Reptilia, 299; Tunicata, 306; Vermes, 314—316
Emery, injection, 267
Encephalon, *see* Neurological methods.
Engelmann, isotonic liquids, 237; epithelium, 453
Enriques, liver of Mollusca, 450
Entire objects, preparation of, 8
Entz, orientation, 87; Protozoa, 477, 478
Eosin, 192; with hæmotoxylin, 231; with methyl green, 193
Eosin and methylen blue, 193
Eosinophilous mixture, 193
Epeira, ova, 312
Epidermis, 331
Epithelium, 331; renal, 372; ciliated, 453
Eppinger, liver, 370
Ergastoplasm, 327, 328
Erhard, glycogen, 320
Erlanger, von, ova of *Ascaris*, 315
Erlicki, liquid of, 43, 378
Ermengem, van, stain for flagella, 483
Ernst, horny tissues, 334
Errera, nigrosin, 181
Erythrosin, 192
Essences, *see* Oil, and Clearing.
Eternod, paraffin blocks, 89
Ether, for narcotisation, 14, 317; for preserving, 5
Eucain, 16
Euparal, 247
Everard, Demoor, and Massart, hæmatoxylin and eosin, 232
Ewald, capillary siphon, 4; section-washing apparatus, 4; blood, 360
Examination and preservation media, watery, 234—238; mercurial, 239; various, 239–241; glycerin, 241; jellies, 242; resinous, 244–248; high refractive liquids, 243, 244
Exner, medullated nerve-fibres, 416
Eycleshymer, celloidin imbedding, 106, 111, 112
Eyes, of Mollusca, 451, 452; of Arthropods, 457; of Asteroidea, 468; of Turbellaria, 467

F.

Fabre-Domergue, syrup, 238; glucose medium, 240; Protozoa, 477, 478
Fairchild, washing cylinders, 4
Fajerstajn, nerve-endings, 335; hæmatoxylin nerve-stain, 436; Golgi's nerve-stain, 429; silver nerve-stain, 436
Fano, neuroglia, 441
Farrants, mounting medium, 239
Fast blue, 195
Fast green, 194
Fat, 71, 351 *et seq.*
Fatty bodies, blackening by osmic acid, 352

The numbers refer to the pages.

Fauret-Fremiet, mitochondria, 475

Faussek, ova of Cephalopoda, 307

Fecundation, artificial, 287

Federici, double imbedding, 115

Feist, methylen blue, 207, 208; spinal cord, 383

Felizat and Branca, thionin, 180

Ferreri, decalcification, 283

Ferria, elastic tissue, 347

Ferric alum, 157

Ferricyanide of potassium for bleaching, 31

Ferrocyanide of copper, injection, 259

Fettponceau, 352

Fibrin, stains for, 367, 368

Fick, Golgi's impregnation, 430; kresyl violet, 197; ova of Axolotl, 301; keratohyalin, 333

Fieandt, neuroglia, 441

Fiedler, *Spongilla*, 474

Field and Martin, paraffin imbedding, 82, 87; double imbedding, 114

Fiessinger, glycogen, 320

Finotti, Marchi's nerve stain, 415, myelin, 418; axis-cylinder stain; 385

Fischel, staining, 137, 138; embryos of duck, 297

Fischer, A., *Fixirung, Färbung und Bau des Protoplasmas*, 21; theory of fixation, 21—24; ditto of staining, 129; hæmatein stains, 160; methyl green, 170, glycogen, 320; nucleoli, 325; Spiegelfärbung, 326; bioblasts, 328

Fischer, B., injection, 268; fuchselin, safranelin, 348

Fischer, E., gold method, 220; nerve and muscle, 338

Fischer, H., glycerin jelly, 243

Fischer, P. M., soap-imbedding, for Trematodes, 465

Fischler, fat, 351, 353

Fish, oil of thyme, 72; celloidin imbedding, 105, 111, 112; decalcification, 281; hardening nervous tissue, 377, 381, 382; Golgi's impregnation, 427; picro-sublimate, 50

Fixation, 2, 19, 28 *et seq.*; by injection, 373; of marine animals, 26; embryos, 288

Fixation images, 21

Fixation precipitates, 21

Fixing agents, action of, 19, 20, 134; characters of the usual, 22; choice of, for beginners, 24; the various, 28—67; cytological, 321; embryological, 288; nerve centres, 373, 375

Flagella, 481

Flagellata, 481

Flatau, hardening brain, 377; Golgi's sublimate method, 433

Flattening sections, 97

Flechsig, gold method, 222; nerve-stain, 414; Golgi's impregnation, 433

Flemming, preservation, 5; acetic acid, 53; chromo-acetic acid, 35; chromo-aceto osmic, 35; action of bichromate, 40; picro osmic acid, 59; safranin, 177, 178; dahlia, 180; connective tissue, 345; Orange method, 189; epithelium, 331; bone, 356; eyes of Gastropoda, 451; injection of Acephala, 452; nucleoli, 194

Flesch, chromo-osmic acid, 35; Weigert's nerve-stain, 409; inner ear, 447; blood, 360; mounting medium, 244

Flint, injection, 269

Florman, celloidin, 108

Floscularïæ, 16

Floyd, ganglia of *Periplaneta*, 457

Fluorides, 408, 410

Foà, fixing mixture, 51; staining mixture, 232

Foettinger, narcotisation, 15

Fol, narcotisation, 18; treatment of osmic material, 30, 31; chromo-aceto-osmic acid, 36; nitric acid,

39; perchloride of iron, 53; picro-chromic acid, 59; vacuum imbedding, 86; serial sections, 125; gold impregnation, 225; iron stain, 227; orseille, 229; injections, meta gelatin, 264; injections, carmine, 260; injections, Berlin blue, 262; injections, brown and yellow, 262; maceration, 272; reconstruction of sections, 291; ova of *Ascaris*, 315; Tintinnodea, 478; absolute alcohol, 61
Foot and Strobell, section cutting, 93
Formaldehyde, 63; as a mordant, 176; for maceration, 272; for nervous tissue, 376—378; for the Golgi impregnation, 427; and *see* Formol.
Formalin, *see* Formaldehyde.
Formalose, *see* Formaldehyde.
Formic acid carmine, 145
Formol, generalities, 63; for hardening, 63, 64, 376—378; for fixing, 63, 64; mixtures, 65—67; for hardening celloidin, 108; as an imbedding mass, 118; as a reducing agent, 64, 225; as a mordant, 176; for gelatin imbedding, 101; for nerve centres, 376; for the Golgi impregnation, 427
"Formol-Müller," 66, 380
Fowl, embryology of, 295—298
Francotte, vacuum imbedding, 86; section-stretcher, 94; hæmatoxylin, 159; ova of *Polyclads*, 314; albumen fixative, 122
Fraenkel, myelin stain, 414; glycogen, 320
Frankl, imbedding box, 79; injection, 258
Freeborn, connective tissue, 344; nerve tissue, 384; picronigrosin, 344
Freezing section method, 117, 374
Frenkel, palladium chloride, 52
Frenzel, mercuro-nitric mixture, 48
Freud, maceration, 275
Frey, H., artificial serum, 238; white injection, 262
Friedenthal, injection, 263; fixative, 57
Friedlaender, Golgi's impregnation, 420; cupric sulphate fixation, 473
Fröhlich, picraminic acid, 188
Fuchselin, 348
Fuchsin, basic, 130, 181; acid, 130, 183
Fuchsin, carbolic, 181; resorcin, 348
Fuchsin S., 183
Fuhrmann, imbedding, 86; celloidin, 105
Funck, honing knives, 91
Fürst, bleaching, 285
Fusari, cartilage, 358
Fuss, acetone, 63

G.

Gad and Heymans, polarisation, 417
Gage, picric alcohol, 58; clearing mixture, 73; section-stretcher, 94; celloidin sections, 110, 112, 126; albumen fluid, 239; starch injection, 268; maceration, 272, 274, 275; decalcification, 282; reconstruction, 291; glycogen, 320
Galeotti, *intra vitam* staining, 137; neutral red, 191
Galesescu, neuroglia, 442
Gallein, 414
Galli, neuroceration, 405
Gamboge injection, 266
Gandolfi, double imbedding, 115
Garbini, safranin, 178; Alcyonaria, 472
Gardiner, ova of *Polychoerus*, 314
Garlic water, 123
Garnier, ergastoplasm, 327; picroformol, 65
Gaskell, gelatin imbedding, 101
Gastric glands, 370

The numbers refer to the pages.

Gastropoda, 17, 449—454; embryology, 307; eyes, 451

Gaudlitz, pyronin, 183

Gaule, fixing liquid, 47; serial sections, 119

Gaultheria, oil of, 72

Gavazzeni, trichohyalin, 333

Gay-Lussac, table for diluting alcohol, 60

Geberg, gold method, 223; corpuscles of Herbst, 334

Gebhardt, crystalline, 336

Gedoelst, digestion, 277; medullated nerve, 406

Gehuchten, van, acetic alcohol, 55; sectioning nervous system, 383; Nissl's stain, 386, 387; Golgi's impregnation, 423, 428; nerve-endings, 447

Gelatin, imbedding, 100; freezing mass, 118; section fixative, 121, 125; injection masses, 257—264; injection masses, carmine, 259—261; injection masses, blue, 261, 262; other colours, 262; mounting media, 242, 243

Gelatin cement, 250

Gemelli, rejuvenating mixture, 427; flagella, 482

Gemmation of Ascidians, 306

Gemmill, imbedding, 86

Gentian blue, 195

Gentian violet, 178

Geoffroy, gelatin medium, 243

Gephyrea, 461

Gerhardt, embryological methods, 299

Gerlach, J., carmine injection, 261; gold method, 224

Gerlach, L., glycerin jelly, 100; embryology of birds, 295; nerve-endings in muscle, 337

Gerota, formol, 64, 378; silver impregnation, 217; Golgi's impregnation, 428

Gerould, *Caudina*, 467

Giacomo, guanin, 321

Giemsa, stain, 365, 480

Gierke, impregnation, 213, 227; maceration, 273; anilin, blue-black, 384; eosin, 232

Giesbrecht, clearing, 69; imbedding trays, 78; squares, 79; paraffin imbedding, 83; section grinding, 117; Copepods, 454

Gieson, van, origanum oil, 72; anilin oil, 111; picro-Säurefuchsin, 188, 232; Säurefuchsin with hæmatoxylin, 232, 385; formalin for nerve-tissue, 377

Giglio-Tos, blood, 363

Gilbert, myelin stain, 407

Gilson, mercuro-nitric fixing fluid, 49; zinc chloride fixing fluid, 53; acetic alcohol with sublimate, 55; nitrate of copper, 57, 67; bleaching bichromate material, 42, 285; rapid celloidin method, 112; mercurial examination liquid, 239; glycerin jelly, 243; sandarac mounting media, 247

Glands, 368—372

Glass, refractive index, 235

Glaue, fixation of *Ascaris*, 463

Glochidia, 309

Glucose mounting media, 240

Glue, marine, 251

Glycerin and alcohol mixtures, 241

Glycerin and gum, 239, 240

Glycerin ether, 349

Glycerin injections, 265, 266

Glycerin jellies, for imbedding, 100, 101; for mounting, 242, 243

Glycerin, refractive index, 235, 241; mounting media, 241—243; extra refractive, 241; method of mounting in, 241, 249, 250

Glychæmalum, 162

Glycogen, 320

Goadby's fluids, 239

Goblet-cells, 369

Goette, hardening ova, 303

Gold chloride, impregnations, 218; commercial salts, 219; pre-impregnation, 219; post-impregna-

The numbers refer to the pages.

tion, 219, 224; marine animals, 225; preserving preparations, 225; and *see* the names of authors.
Gold size, 249, 251
Gold toning baths, *see* Toning baths.
Gold, colloidal, 401
Goldmann, *intra vitam* stains, 138
Goldscheider and Flatau, Nissl's stain, 387
Golgi, chromate of silver impregnation: introduction, 418—421; slow process, 421—423; rapid process, 423—425; mixed process, 425; variations, 426—431; gold method, 224; fixation by injection, 374; bichromate and sublimate method, 431; intracellular net, 403; neuroceratin, 404
Golgi, corpuscles of, 340
Gollman, *intra vitam* stains, 138
Golodetz, fat, 352
Golodetz and Unna, iron stain, 227; manganese stain, 227; cholesterin, 333
Golovin, hæmatoxylin, 167
Golovine, neutral red, 192
Gordon, stain for flagella, 483
Goronowitsch, embryology of Salmonidæ, 305
Gothard, Nissl's stain, 387
Gräberg, stain, 191
Graefe, solvents of paraffin, 81
Graf, chromic mixture, 35; formol, 65; Hirudinea, 460
Graff, von, Turbellaria, 466
Graham, Trichinæ, 463
Gram, staining method, 179
Grandry, corpuscles, 334
Grand-Moursel and Tribondeau, pancreas, 370
Grandis and Mainini, lime salts, 320
Granule cells, 349—351; and *see* Leucocytes.
Granules, 327
Grape-sugar imbedding, 115
Graphic or plastic reconstruction, 289; and *see* Orientation.
Graser, staining methods, 172, 181
Grassi, hæmatozoa, 479
Gray, inner ear, 447
Greef, methods for the eye, 442
Gregarinæ, 478, 479
Grenacher, alum carmine, 142; borax-carmine, 148; hydrochloric acid carmine, 149; hæmatoxylin, 163; purpurin, 229; castor oil for mounting, 248; bleaching mixture, 285; eyes of molluscs, 451
Greppin, Golgi's impregnation, 430
Grieb, alum-carmine, 143
Griesbach, Congo red, 191; benzopurpurin, 191; Bengal rose, 192 iodine green, 194; elastic tissue, 347; blood, 360
Grinding sections, 115
Grönross, ova of *Salamandra*, 302
Groselj, Actinida, nervous system, 471
Grosser, injection, 267
Groot, de, serial sections, 119; iron carmalum, 146
Grübler and Hollborn, address, 11; anilin blue-black, 196; salts of gold, 220
Grünpulver, 170
Grünstein, bladder of frog, 343
Grünstichblau, 195
Grynfeltt and Mestrezat, bleaching, 285
Guanin, detection of, 321
Gudden, Pal's nerve-stain, 411; Golgi's, 429
Gudernatsch, serial sections, 121
Gudger, embryological methods, 304
Guéguen, methyl salicylate, 72
Guernsey blue, 195
Guieysse, double stain, 194; goblet cells, 369
Guignet, injection, 262
Gulick, ova of *Heterakis*, 316
Gulland, serial sections, 119, 124; blood, 359, 361, 362; formol, 65

The numbers refer to the pages.

Gum, imbedding, 115; for freezing, 117, 118; mounting media, 239, 240; injection mass, 267; mucilage, 118; for labels, 484
Gum damar, 235, 246
Gum sandarac for mounting, 248
Gum Thus, 248
Gurwitsch, hæmatoxylin, 158
Guyer, ova of Amphibia, 300
Gymnotus, 340

H.

Hadži, Hydroidea, 473
Haecker, micro-chemistry of the cell, 321; Cladocera, 313
Hæmacalcium, 165
Hæmalum, 161, 162, 165
Hæmalum and indigo-carmine, 230
Hæmastrontium, 165
Hæmateate of ammonia, 154
Hæmatein, generalities, 152—154; formulæ for stains, 155 *et seq.*: and *see* Hæmatoxylin.
Hæmatoxylin, generalities, 152; alcoholic stock solution, 153; Unna's solution, 162, 164; characters of alum-hæmatoxylin stains, 159; formulæ for stains, 155 *et seq.*; the iron compounds, 155—159; other compounds, 159 *et seq.*
Hæmatoxylin and eosin, 231; and picro-Säurefuchsin, 232; and safranin, 232; and Säurefuchsin, 232, 385; combination stains, 231
Hæmatozoa, 479
Haensel, liquid of, 59
Hahn, cooling paraffin, 88
Hair, 333
Malle and Born, celloidin imbedding, 106
Haller, Béla, maceration, 275
Hamann, Acanthocephali, 462; Asteroidea, 468; Chilopoda, 455
Hamburger, ova of *Argyroneta*, 313; salt solution, 237; injection, 267
Hamilton, freezing method, 117; hardening brain, 380
Handwerck, osmicated fat, 352
Hansen, hæmatoxylin, 159, 163, 166; picro-säurefuchsin, 188; iron cochineal, 146; eosin, 192
Häntsch, glycerin liquid, 242
Hardening, generalities, 26; practice of, 27; nerve-centres, 374
Hardening agents, *see* Fixing agents.
Hardy, Rotatoria, 461
Hári, mucin, 368
Harmer, silver impregnation, 218
Harris, hæmatoxylin, 348; toluidin blue, 163, 180, 210; methylen blue, 209; thionin, 210; nerve-stain, 414; encain, 16; elasthæmatein, 348
Harting, calcium chloride, 238; white injection, 262; gamboge injection, 266
Harvey, artificial parthenogenesis, 287
Haswell, dehydration apparatus, 3; embryology of Cestodes, 314
Hatschek, *Amphioxus*, 305
Havet, *Distoma*, 466; *Metridium*, 471
Haug, decalcification, 280, 282, 283; nerve-stain, 414
Hayem, blood, 361
Heat, for killing, 12
Heckert, ova of Trematodes, 315
Heidecke, paraffin imbedding, 85
Heidenhain, M., sublimate solution, 47; paraffin imbedding, 82, 98; serial sections, 119, 123; iron-hæmatoxylin, 157, 326; Ehrlich-Biondi stain, 184, 185, 186; Bordeaux R., 326; vanadium hæmatoxylin, 167; Cœrulein S., 194; Blauschwarz, Brillantschwarz, 191, 196; "Subtriessig," 56; glycerin jelly, 243; salicylic acid, 56; neutral dyes, 132; Congo Corinth, 191; Benzo purpurin, 191.
Heidenhain, R., chromic hæmatoxylin, 166; Ehrlich-Biondi stain, 184

The numbers refer to the pages.

Heider, paraffin sections, 96

Heine, phosphorus, 320

Heinke and Ehrenbaum, pelagic ova, 305

Heinrich, connective tissue, 346

Held, sublimate solution, 48; stain for nerve-cells, 388; iron hæmatoxylin, 159; formol mixture, 67; section cutting, 93

Helix, 16, 307, 424, 450

Heller and Gumpertz, medullated nerve, 416

Helly, fixing mixture, 51; serial sections, 121

Henchman, ova of *Limax*, 308

Henking, section cutting, 96; embryology of Arthropoda, 309, 310, 312; examination liquid, 318

Henneguy, overstains, 142; section-fixing, 120, 123; alum-carmine, 143; permanganate method, 176; embryological methods, 291—294, 296, 297, 300, 303, 307; Protozoa, 476; re-staining old mounts, 7

Hennings, Chilopoda, 455; eyes of Arthropods, 457

Hénocque, gold method, 222

Herbst, corpuscles, 334, 392; Crustacea, 456

Herla, stain for ova, 172

Hermann, platino-aceto-osmic acid, 38, 322; safranin and gentian stain, 179; osmic acid and pyroligneous acid stain, 226; cytological methods, 326; papillæ foliatæ, 335

Herrick, ova of *Astacus*, 313

Hertwig, silver impregnation, 216, 218; maceration, 273; Medusæ and Actiniæ, 273, 473; ova of *Triton*, 301; ova of *Rana*, 302

Herxheimer, plasma fibrils, 332; Kresylviolet, 197; fat, 352, 353; elastic tissue, 347

Herzog, retina, 444

Heschl, amyloid degeneration, 171

Hesse, Heteropoda, 452; eye of *Pecten*, 452

Hessert, flagella, 483

Heterakis, ova, 316

Heteropoda, eyes, 451

Heurck, van, mounting medium, 244

Heydenreich, amber varnish, 253

Heyder, embryos of *Arion*, 308

Heymans, Cephalopoda, 450

Heymons, embryology of Blattida, 311

Hickson, Brazilin, 228, 229; eosin and hæmatoxylin, 231; maceration, 276; eyes of *Musca*, 457

Hill, A., nerve-stain, 413; Golgi's impregnation, 425, 428

Hill, E. C., embryological method, 291

Hindle, Hæmatozoa, 480

Hippel, retina, 444

Hirota, egg of fowl, 298

Hirudinea, 16, 206, 397, 460

Hirschfelder, Rotifers, 461, 462

Hirschler, ova of *Donacia*, 311

His, nitric acid fixation, 39; impregnation, 213; embryos of Selachia, 305

Hochstetter, injection, 268

Hoehl, bichromate and osmic acid, 38; digestion, 277

Hofer, hydroxylamin, 16

Hoffmann, E. H., blastoderm of birds, 297

Hoffmann, F. W., vacuum imbedding, 85

Hoffmann, R. W., stain for chitin, 456; orientation, 87

Hofmann, K., Cercariæ, 466

Hofmann, M., injection, 267

Hofmann's Grün, 194

Hoggan, histological rings, 214; iron stain, 227; silver nitrate, 216

Holl, imbedding, 81

Hollande, serial sections, 123 fixation of Tracheata, 455

Holmes, ova of *Planorbis*, 308

The numbers refer to the pages.

Holmgren, fixatives, 56
Holothurioidea, 18, 467
Homarus, embryology, 313
Honing knives, 91
Hopewell-Smith, odontoblasts, 355
Hopkins, maceration, 274
Horn, 333
Hornowski, connective tissue, 346
Hoskins, embryological method, 297
Houser, myelin stain, 407
Hoyer, silver impregnation, 216; gold impregnation, 222; mounting medium, 240; carmine-gelatin injection, 260; blue gelatin injection, 262; yellow gelatin injection, 262; green gelatin injection, 262; shellac injection, 268; oil-colour injection, 268; mucin, 368; fixing mixture, 51
Hoyer, jun., formol, 64; imbedding, 80
Hrdlicka, hardening brain, 378
Huber, Golgi's impregnation, 430; injection, 269
Hudson, Rotifers, 461
Hyatt, shellac imbedding, 115
Hydra, 16, 202, 472, 473
Hydrate of chloral, *see* Chloral.
Hydrochloric acid, for decalcification, 280, 282; for maceration, 275
Hydrochloric acid alcohol, 62
Hydrochloric acid carmine, 149
Hydrofluoric acid, 283, 284
Hydrogen peroxide, for narcotisation, 18; for bleaching, 30, 33, 285
Hydroidea, 18, 472
Hydroxylamin, narcotisation, 16
Hymenoptera, 311
Hypertonic liquids, 236
Hypochlorite of potash, 278, 285
Hypochlorite of soda, 278, 285
Hyposulphite fixing baths, 217, 393, 398, 400
Hypotonic liquids, 236

I.

Ide, double imbedding, 114; epithelium, 331
Igacuschi, liver, 371
Ijima, embryology of Planaria, 314
Ikeda, section-fixing, 123
Ilberg, Nissl's stain, 388
Imbedding, defined, 6; small objects, 79, 289, 478
Imbedding methods, 75; manipulations, 76 *et seq.*; trays, thimbles, 76; boxes, 78; trough, 79; *in vacuo*, 85; paraffin, 81 *et seq.*; soap, 100, 465; gelatin, 100; celloidin (collodion), 102
Immobilisation of small organisms, 461, 476
Impregnation defined, 212; positive and negative, 212; primary and secondary, 212
Impregnation methods, 212; silver, 214—218; gold, 218—225; other metals, 226, 227
Indamin blue, 385
Index of visibility, 234, 245
Indian ink injection, 267, 458
Indices of refraction, 234, 235
Indifferent liquids, 235 *et seq.*
Indigen, 195
Indigo, 229
Indigo carmine, 229; for injection, 266
Indigo substitute, 195
Indophenol, 353
Indulin, 195
Indulin-aurantia-eosin, 193
Infusoria, 475 *et seq.*
Injections, 257; gelatin, 257—264; other masses, 264—268; natural, 269, 459, 460; of Mollusca, 452; of Arthropods, 458; of Hirudinea, 460
Inner ear, 445
Insabato, connective tissue, 346
Insects, *see* Arthropoda.
Instruments, 9
Intercellular bridges and canals, 331
Intestine, 370
Intracellular network of Golgi, 403, 404
Intra vitam staining, 136, 201, 202, 203

The numbers refer to the pages.

Inversion stains, 135

Iodate of sodium, 32

Iodide of potassium, 46, 53, 271

Iodine, for removing sublimate, 46; for fixing, 53; for hardening, 379; Lugol's solution, 53

Iodine green, 194

Iodised serum, 237, 238; maceration in, 271

Iridium chloride, 52

Iris, 342

Iron alum, fixative, 53; mordant, 157

Iron, ammonio-sulphate, 157

Iron, detection of, 320

Iron, impregnations, 227

Iron perchloride, *see* Perchloride.

Iron, pyrogallate, 227

Iron-Brazilin, 229

Iron-carmine, 145, 146

Iron-cochineal, 146

Iron-hæmatoxylin, 155—159, 407

Isotonic liquids, 235 *et seq.*

Israel, Ehrlich-Biondi stain, 186; acidophilous mixture, 193; orcein, 229

Iwanzoff, electric organs, 340; nematocysts, 470; Holothurids, 468

J.

Jackson, clearing, 69

Jacobs, freezing mass, 118

Jacoby, bleu de Lyon, 195

Jäderholm, neurofibrils, 402

Jaenichen, *Planaria*, 467

Jäger, glycerin liquid, 242

Jakimovitch, silver impregnation, 217

Jander, bleaching, 286

Janssens, iron hæmatoxylin, 159; bleu carmine, 196

Janus green, 194

Japanese section-fixing method, 123

Jaquet, leeches, 460; *Lumbricus*, 459

Javelle, eau de, 278, 285

Jelgersma, anilin blue-black, 384

Jelinek, picric acid, 57; "Stabilit," 110

Jellies, *see* Glycerin.

Jenner, blood, 364, 366

Jennings, Rotatoria, 314

Jensen, Infusoria, 476

Joest, Annelids, 458

Johne, freezing, 117

Johnson, Lindsay, fixing mixture, 38, 54; cement for collodion blocks, 109; sunning metallic solutions, 213; gold impregnation, 223; retina, 443

Johnston, reconstruction, 291; paraffin mass, 99; picronigrosin, 384

Johnstone-Lavis, section grinding, 116

Joliet, gum imbedding, 115

Jonescu, brain of bees, 457

Jordan, clearing agents, 69, 112; imbedding box, 79; imbedding, 114; orienting, 87; cutting, 96; serial sections, 125

Joris, neurofibrils, 401

Joseph, silver impregnation, 213; injection, 267; eleidin, 333

Joubin, Nemertians, 464

Julien, flagella, 483

Juliusburger, stain for nerve-cells, 388

Jung, R., microtomes, etc., address, 10; knife-holders, 93; freezing, 117

K.

Kadyi, soap imbedding, 100; nerve-stain, 384; hardening brain, 378

Kaes, nerve-stain, 413

Kaiser, sublimate solution, 45; glycerin jelly, 100; Bismarck brown, 181; nerve-stains, 384, 412; Acanthocephali, 463

Kalb, stain for *Spirochæte*, 483

Kallius, Golgi's impregnation, 426, 428, 431; cartilage, 357

Kaplan, neuroceratin, 405; axis cylinder stain, 437; myelin stain, 418

Kappers, cooling paraffin, 88; elderberry stain, 385
Kappers and Ketjen, nerve-tissue, 384
Karawaiew, Protozoa, 478, 479; *Anobium*, 312
Kastschenko, reconstruction, 291; embryos of *Raja*, 305
Kato, glycogen, 320; silver stain, 397
Katz, inner ear, 446
Keibel, embryology of *Sus*, 294
Kemp, blood-platelets, 367
Kendall, flagella, 482
Kent, fixative, 53
Kenyon, phospho-molybdic hæmatoxylin, 167; Pauropoda, 455; brain of bees, 457
Keratohyalin, 333
Kernschwarz, 228
Kerr, orientation, 86; serial sections, 128; reconstruction, 290
Kerschner, gold method, 221
Kidney, 372
Killing, generalities, 12; various processes, 12—18
King, ova of *Bufo*, 302; hardening brain, 381
Kingsbury, fat, 353
Kingsley, ova of *Limulus*, 313
Kionka, egg of fowl, 298
Kishi, inner ear, 446
Kinshinouye, embryology of Araneida, 312; of *Limulus*, 313
Kissling, melting-points of paraffin, 99
Kizer, blood, 361
Klebs, glycerin jelly, 100
Klein, chromic acid, 34; cornea, 335; intestine, 44
Kleinenberg, picro-sulphuric acid, 58; hæmatoxylin, 164
Klingmüller and Veiel, sublamin, 51
Knife position, 89—93
Knife-holders, 92
Knives, microtome, 90; honing, 91
Knowen, orientation, 87
Koch, von, section method, 115
Kockel, fibrin, 368
Kodis, molybdic hæmatoxylin, 167; myelin stain, 407; hardening nerve-tissue, 381
Koerner and Fischer, flagella, 482
Kofoid, embryology of Gastropoda, 308
Koganei, iris, 342
Köhler, Tæniæ, 465
Kohn, fixing mixture, 51
Kölliker, ova of rabbit, 292, 294; bone, 356
Kollmann, fixing ova, 304
Kolmer, epiderm of *Lumbricus*, 392; fixation, 25; retina, 444
Kolmer and Wolff, imbedding, 86
Kolossow, osmic mixtures, 32; gold method, 223; osmic acid stain, 227; epithelium, 331
Kolster, gastric glands, 370; dehydration, 4; imbedding, 86; mitochondria, 330
Königstein, maceration, 275
Kopsch, embryology of Salmonidæ, 304; Golgi impregnation, 427; Golgi network, 404; eyes of Cephalopods, 452; blood-plates, 367
Korotneff, narcotisation, 14
Korschelt, embryology of *Loligo*, 307; Protozoa, 477
Kossinski, stain, 195
Kostanecki and Siedlecki, ova of *Ascaris*, 316; sublimate mixture 49
Kostanecki and Wierzejski, ova of *Physa*, 309
Kotlarewsky, ganglion cells, 381
Kowalewsky, fishes, embryology, 304
Kowalski, neurofibrils, 397
Kozowsky, myelin stain, 412
Krassuskaja, injection, 269
Krause, R., Ehrlich-Biondi stain, 184; freezing, 117; injection, 261; thiophen green, 194; liver, 371; retina, 445; salivary glands, 369; corpuscles of, 335; section cutting, 93

Krauss, silver impregnation, 217
Kreasote, refractive index, 235; for clearing, 73, 111
Krecker, chloreton, 15
Krosofuchsin, 181
Kresyl violet, 197, 333
Kriegbaum, washing apparatus, 4
Krooh, Mentz von, stain for nerve-cells, 387
Krohnthal, lead impregnation, 434
Kromayer, plasma fibrils, 332; fibrin stain, 368
Kronecker's serum, 237
Krönig, cement, 252
Krueger, embryology of Harpactida, 313
Krysinsky, photoxylin, 103
Kühne, H., freezing method, 118
Kühne, W., maceration, 274; digestion, 277
Kuhnt, retina, 445
Kükenthal, narcotisation, 16, 459; blood-vessels, 459; intestine of *Lumbricus*, 458
Kull, goblet cells, 369
Kultschitzky, preservation, 5; fixing liquids, 43, 44; double imbedding, 114; tactile corpuscles, 334; hæmatoxylin nerve-stain, 412; neuroglia, 442; elastic tissue, 371; mucus cells, 369; spleen, 371
Kupffer, axis-cylinder stain, 389; liver, 371
Kuskow, digestion, 277

L.

Labarraque, eau de, 278, 285
Labyrinth, 445—447
Lachi, formol for nerve-tissue, 377
Lactate of silver, 216, 429
Lactic acid, 280, 283
Lactophenol, 241
Laffont, keratohyalin, 333
Lakes, 134
Lamellibranchiata, 274, 286, 449—454; embryology, 309; eyes, 452
Lambert, ova of *Epeira*, 312
Landois, impregnations, 227; maceration, 272
Landolt, retina, 445
Lang, liquid of, 48; *Helix*, 450
Langdon, nervous system of *Nereis*, 460
Lanoe, de, Marchi's stain, 415
Lanoerhans, mounting medium, 240; tactile corpuscles, 334
Lankester and Bourne, eyes of *Limulus*, 457
Lansberg, Protozoa, 477
Larvæ of Amphibia, 317; of sponges, 475
Laslett, nerve-stain, 412
Lattice fibres, 371
Laurent, methylen blue and eosin, 193; orcein, 229
Lauterborn, Protozoa, 79, 478, 481
Lauth, violet, 179
Lavdowsky, formol mixture, 65; chromo-platinic mixture, 40; bichromate and sublimate, 44; methylen blue, 206; chloral preservative solution, 238; sandarac for mounting, 248; cochlea, 447; maceration, 276
Laveran, blood, 366; bleu Borrel, 366
Law, teeth, 356
Lawrence, glycerin jelly, 242
Lead acetate, 381
Lead chromate, impregnation, 227
Lead sulphide, impregnation, 227, 434
Leber, impregnations, 227; retina, 443
Lebrun, ova of Amphibia, 300
Lecithin, 351
Lee, A. B., preservation of material, 5; dissections, 8; lemon-juice for fixing, 13; narcotisation, 17, 18; keeping osmic acid, 28; bleaching osmic material, 31; making up chromoaceto-osmic, 36; nitric acid for fixing, 39; sublimate solution, 45; picro acetic acid,

58; formol, 64; cedar oil, 5, 70, 82; 83; oil of turpentine, 72, 81; paraffin imbedding, 82, 83; paraffin masses, 98; celloidin imbedding, 104, 106, 108, 113; Mayer's albumen, 122, 125; serial sections, 119; stains recommended, 140, 384; Ehrlich - Biondi stain, 186; gentian and orange stain, 190; neutral red, 192; gold impregnation, 222, 225; *intra vitam* staining, 137; iron - carmine, 145; iron-hæmatoxylin, 155, 156; bleu de Lyon, 195; safranin, 169, 177; stains for nerve-tissue, 384; toluidin blue, 180; Säurefuchsin, 183; osmic acid and pyrogallol, 226; Kernschwarz, 228; glycerin liquid, 242; desilification, 284; cedar oil for mounting, 245; paper cell mounting method, 250; cytological methods, 318, 321 *et seq.*; Alcyonaria, 472; Hirudinea, 460; Nemertina, 464; hardening nerve-centres, 377; sponges, 284, 474; neuro-fibrils, 391, 398, 399; myelin stain, 407; Golgi impregnation, 425; retina, 442, 444; *Pyrosoma*, 448; Opisthobranchiata, 450

Leeuwen, van, fixation of Hexapods, 455

Lefevre, imbedding trough, 79

Legal, picro-alum carmine, 145

Legendre, intracellular network, 404; nervous system of Pulmonata, 451

Léger, Sporozoa, 479

Legros, silver impregnation, 217; *Amphioxus*, 305

Leishman, stain for blood, 366

Lemon-juice for fixing, 13

Lemons, oil of, 235

Lendenfeld, von, section cutting, 93; sponges, 474

Lendvai, honing knives, 91

Lennhoff stain for nerve-cells, 387; methylen blue impregnation, 436; iron stain for axis cylinders, 436

Lenhossék, von, cœrulein, 194; stains for nerve-cells, 388; Golgi method, 424; eyes of Cephalopods, 452; nerves of Annelids, 460; tongue of rabbit, 335; picro - sublimate, 50; platino-sublimate, 52

Lennox, retina, 445

Lens, crystalline, 336

Lenssen, Rotatoria, 314, 462

Leontowitsch, methylen blue, 209

Lepidoptera, embryology, 310

Lepkowsky, teeth, 355, 356

Leuckhart, imbedding boxes, 78

Leucobases, 202

Leucocytes, *see* Blood and Granules.

Leutert, lime salts, 320

Levaditi, blood, 363; stain for *Spirochæte*, 483

Levi, nucleoli of nerve-cells, 325; connective tissue, 346

Levulose for mounting, 241

Lewis, Bevan, anilin blue-black, 196, 384; hardening brain, 379; staining ditto, 384

Lewis, M., nerves of Annelids, 460

Lewy, Marchi's stain, 415

L'hermitte and Guccione, neuroglia, 442

Lichtgrün, 194

Liebermann, carmine, 141

Liebetanz, stain for flagella, 481

Liesegang, sections of brain, 383; silver stain, 398

Light, action on alcohol with chromic material, 33; on metallic salts, 213

Light green, 194

Lillie, embryology of *Unio*, 309

Limax, ova, 308

Lime-water, 272

Lime salts, detection of, 320

Limulus, embryology, 313

Linaloa, oil of, 112

The numbers refer to the pages.

LINDSAY JOHNSON, *see* JOHNSON.
Linseed oil injection, 268
LINTWAREW, acetone, 63
LINVILLE, ova of *Limax*, 308
Liquid of Müller, of Erlicki, of Merkel, etc., *see* the names of the respective authors.
Liquidambar, 248
Liquor ferri sulphurici oxidati, 156
LIST, methyl green and eosin, 193; hæmatoxylin and eosin, 231; goblet-cells, 369; Coccidæ, 456; Actinida, 471; nucleoli, 325; Mytilidæ, 450.
Lithium carmine, 147
LITTLE, nematocytes, 470
Liver, 370; of Mollusca, 450
Lo BIANCO, tobacco narcotisation, 13; alcohol narcotisation, 14; chloral narcotisation, 15; poisoning method, 17; acetic acid, 54; chromo-acetic acid, 35; chromo-osmic acid, 35; picro-chromic acid, 59; osmic acid and bichromate, 37; sublimate solution, 45; chromic sublimate, 50; acid alcohol, 62; chromo-formol, 67; methods for marine animals, 448 *et seq.*; Alcyonaria, 472; Asteroidea, 468; Brachiopoda, 449; Bryozoa, 449; Chætopoda, 459; Crinoidea, 469; Ctenophora, 474; Echinoidea, 468; Gastropoda, 450; Gephyrea, 461; Holothurioidea, 467; Lamellibranchs, 449; Mollusca, 449, 450; Nematoda, 463; Nemertina, 464; Ophiuridea, 469; Protozoa, 478, 479; Siphonophora, 473; Trematodes, 465; Tunicata, 448; Zoantharia, 472
LOCKE, salt solution, 237
LOCY, embryology of Araneida, 312
LOEWENTHAL, liquid of Erlicki, 43; carmine, 149
LOEWY, epidermis, 331
LÖFFLER, stain for flagella, 481
LOISEL, *intra vitam* staining, 138; neutral red, 191; fat and lecithin, 351, 352
LONGHI, Protozoa, 478
LONGWORTH, corpuscles of Krause, 335
LÖNNBERG, *Triænophorus*, 465
LOOSS, eau de Labarraque, 278; Nematodes, 463; *Bilharzia*, 465,
LORD, stain for nerve-cells, 387
LÖWIT, gold method, 220; blood, 361
LOYEZ, myelin stain, 407
LUGARO, neurofibrils, 400, 402
LUGOL, iodine solution, 53
LÜHE, Cestodes, 465
LUITHLEN and SORGO, Nissl's stain, 387
Lumbricus, neurofibrils, 397; epidermis, 424; killing and fixing, 458, 459
LUNDVALL, cartilaginous skeletons, 358
LUSTGARTEN, Victoria blue, 180, 347
LUXENBURG, stain for nerve-cells, 388
Lymphatic glands, 371
LYON, immobilisation of Infusoria, 476
Lysol, for maceration, 276

M.

MAAS, carmine and malachite green, 231; digestion, 277; larvæ of sponges, 475
MACALLUM, carmine and indigo-carmine, 230; impregnation, 213; phosphorus, 320; copper, 320; lime salts, 320; potassium, 321
MACBRIDE, *Amphiura*, 470
Maceration, 270 *et seq.*; of epithelium, 331, 453; of muscle, 342; of Actinida, 471
MACDONALD, impregnation method, 434
Magdala red, 181
Magenta, 181, 230
Magenta S, 183

The numbers refer to the pages.

Magini, zinc impregnation, 436
Magnesia-carmine, 147
Magnesium, chloride or sulphate, narcotisation, 17; peroxide, 285
Mährenthal, osmic acid stain, 227
Maier, serial sections, 126
Malachite green, 194, 231
Malapterurus, 340
Malaria-parasites, 479
Malassez, salt solution, 237
Mallory, eosin and methylen blue, 193; anilin blue and orange for connective tissue, 345; phospho-molybdic hæmatoxylin, 167; phospho-tungstic hæmatoxylin, 167, 348; iron hæmatoxylin, 159; neuroglia, 440, 441
Mammalia, embryology, 291
Manchester brown, 172
Manfredi, gold method, 223
Manganese chloride, 227, 237
Mann, bleaching, 31; celloidin, 104; chromo-sublimate, 50; picro-sublimate, 50, 66; osmio-sublimate, 50; fixing nerve-centres, 374, 381; formol mixtures, 66; hæmatein stain, 164; serial sections, 123; toluidin blue, 180; methyl blue and eosin, 196
Marcacci, maceration, 274
Marcano, blood, 361
Marchi, corpuscles of Golgi, 341; degenerate nerves, 415; mucus of Gastropoda, 450
Marchoux, formol mixture, 67
Marcus, formol for spinal cord, 377, 411
Maresch, connective tissue, 346
Marine animals, precautions in preparing, 26; silver impregnation, 217; gold impregnation, 225
Marine glue, 251
Mark, collodionising sections, 95; wax cutter, 291
Mark and Long, living ova, 293
Marpmann, gum for labels, 484
Marsh, carmine and indigo-carmine, 230; gelatin cement, 250
Martin, benzo-azurin, 181, 197; goblet-cells, 369; injection of tracheæ, 457
Martinotti, C., elastic tissue, 347, 348; Golgi impregnation, 429
Martinotti, G., anilin blue-black, 384; picro-nigrosin, 384; elastic tissue, 347
Martinotti, L., hæmatoxylin, 164; toluidin blue, 211; eosinophilous cells, 367
Martinotti and Resegotti, safranin, 178
Mason, nervous system of reptiles, 382
Masson, connective tissue, 346
Mastzellen, 349—351
Matschinsky, bone, 355
Matuszewski, Marchi's stain, 415
Maurice and Schulgin, bleu de Lyon, 195, 230
Mawas, retina, 445
Maximow, sublimate and bichromate, 51; serial sections, 125; plasma cells, 351
May and Gruenwald, blood, 364
Mayer, A. G., narcotisation, 17
Mayer, P., preparing marine animals, 26; bleaching osmic objects, 31; washing out chromic objects, 33; formol, 64; washing sublimate material, 46; picro-sulphuric acid, 58; picro-nitric, 58; picro-hydrochloric, 59; acid alcohol, 62; paraffin imbedding, 78, 80, 83, 98; water-bath, 85, 88; serial sections, water method, 119; albumen method, 122; section stretcher, 94; theory of staining, 136; staining with carmine, 141; staining with cochineal, 141; carmalum, 144; aluminium chloride carmine stain, 144; alum-carmine, 143; magnesia-carmine, 147; picro-carmine, 147; picro-magnesia-carmine, 148; paracarmine, 149; hydrochloric acid carmine, 149;

alcoholic cochineal, 150; theory of hæmatoxylin staining, 152, 153; hæmatein, 154; hæmateate of ammonia, 154; bluing hæmatein stains, 160; hæmalum, 161, 162; glychæmalum, 162; Ehrlich's hæmatoxylin, 164; hæmacalcium, 165; methyl green, 170; iodine green, 194; triacid mixture, 187; carmine and indigo-carmine, 230; hæmalum and indigo-carmine, 230; mucicarmine, 369; muchæmatein, 369; stain for chitin, 456; decalcification, 281; desilicification, 283; bleaching, 284, 285; injection, 264, 266; mucus, 360; terpinol, 73; orientation, 87, 470; cutting large sections, 95; staining through paraffin, 122; double imbedding, 115; hæmastrontium, 165; glycogen, 320; cartilage, 357; mounting Plutei, 470

MAYER, P., ANDRES, and GIESBRECHT, section-stretcher, 94

MAYER, P., and SCHOEBEL, knife-holders, 93

MAYER, S., neutral red, 192; violet, 196; methylen blue, 205, 207, 210; cornea, 331; connective tissue, 344.

MAYSEL, Bismarck brown, 172

McCLURE, nerve-cells of Pulmonata, 451

McCRORIE, night-blue for flagella, 483

McFARLAND, fixing method, 374

Medullated nerves, structure, 404—406; stains for, 407—418

Medusæ, 17, 273, 473

MEHNERT, embryological methods, 299

MEIROWSKY, stain for *Spirochæte*, 483

MEISENHEIMER, embryology of *Limax*, 308

MEISSNER, corpuscles of, 335; cooling paraffin, 88

MENDEL and BRADLEY, zinc, 320

MERCIER, nerve-stain, 414

Mercuric mixtures, 47 *et seq.*, 239; and *see* Sublimate.

Mercury, bichloride, *see* Sublimate; biniodide, 243; cyanide, 381

MERK, elastic tissue, 347

MERKEL, chromo-platinic mixture, 40; carmine and indigo-carmine, 230

MERKEL and KRAUSE, molybdenum impregnation, 227

MERKEL and SCHIEFFERDECKER, celloidin imbedding, 102

MERTON, eyes of Mollusca, 452

MERZBACHER, neuroglia, 442

Metachromasy, 135

Metagelatin, 264

Metallic salts, action of light on, 213

Metallic stains, 212

METCALF, embryology of *Chiton*, 309

Methyl alcohol, for narcotisation, 15; refractive index, 235

Methyl blue, 196

Methyl green, 170

Methyl green and eosin, 193

Methyl mixture, 275

Methyl salicylate, 72

Methyl violet, 172, 196; test for, 170; progressive stain, 172; regressive, 181

Methyl violet B, 196

Methylal, for dehydration, 4, 208

Methylanilin green, 170

Methylanilin violet, 172

Methylenazur, 199, 200

Methylen blue, chemistry of, 199; uses of, 200; for *intra vitam* staining, 201; for central nervous system, 418, 435; for impregnation, 203—210; generalities, 199; staining nervous tissue, 203—210, 386—388, 414, 418, 435; staining by injection or immersion, 204; diffusion processes, 435; the solutions employed, 204; preservation of the preparations, 207—209; impregnation of epithelia, etc, 210

The numbers refer to the pages.

Methylen blue, polychrome, 199

Methylen blue and eosin, 193

Methylen blue and erythrosin, 388

Methylen red, 199

Metzner, toluidin blue, 181

Meves, liquid of Flemming, 35, 36; imbedding capsules, 80; mitochondria, 328, 330; neuroglia, 441

Meyer, E., celloidin sections, 112, 115; re-staining, 7

Meyer, P., Weigert's myelin stain, 411

Meyer, Semi, methylen blue for nerve-centres, 435; Berlin blue stain, 403

Mibelli, elastic tissue, 347

Michaelis, ova of *Triton*, 301; Janus green, 194; methylen-azur, 199; Scharlach R, for fat, 352; stain for blood, 363; polychrome methylen blue, 200

Michailow, methylen blue, 203, 206, 209

Micro-chemistry of the cell, 319 *et seq.*

Microtome knives, 89—93; microtomes, 9

Migula, glycerised serum, 238

Milk, injection mass, 268

Miller, caoutchouc cement, 249, 251; injection, 262

Minchin, sponges, 474; hæmatozoa, 480

Minervini, elastic tissue, 348

Mingazzini, sublimate mixture, 56

Minnich, Pal's hæmatoxylin, 411

Minot, microtome, 10; celloidin sections, 111; epidermis, 331

Miracidia, 315

Mitochondria, 328, 475

Mitrophanow, double imbedding, 115; Wasserblau, 196; nerve-stain, 413; blastoderm of birds, 297; epidermis, 331; organs of sixth sense, 335

Mitsukuri, embryology of tortoise, 299

Möbius, maceration, 274

Moerner, cartilage, 357

Moleschott, maceration, 272

Moleschott and Piso Borme, maceration, 271

Möller, picro-Säurefuchsin, 189; formol mixture, 66

Moll, cartilage, 357

Mollison, fat, 353

Mollusca, 449 *et seq.*; embryology, 307—309

Molluscoida, 449

Molybdate of ammonium, impregnation, 227, 401

Molybdenum stains for neurofibrils, 401, 402; toluidin blue, 401; thionin, 402

Molybdic hæmatoxylin, 167

Mönckeberg and Bethe, treatment of osmic material, 31, 285

Mondino, medullated nerve, 405

Monobromide of naphthalin, 235, 244

Montanari, neurofibrils, 402

Montgomery, Nemertina, 464; Araneida, 312; nucleoli, 325

Monti, A., copper impregnation, 437

Monti, R. and L., gastric glands, 370

Moore, V. A., freezing method, 118

Mordants, 133 *et seq.*

Moreaux, formol mixture, 66

Morel and Bassal, hæmatoxylin, 159

Morel and Doleris, triacid, 187

Morgan, embryology of Amphibia, 301, 302; of Ascidians, 306; of *Periplaneta*, 309, 311

Morphia, as a vaso-dilator, 258

Morton, flagella, 483

Moseley, shell, 452

Mosse, myelin stain, 417; Nissl stain, 389

Motor nerve-endings, 337 *et seq.*

Mounting in fluids, 249, 250

Mounting media, *see* Examination and Preservation.

Mozejko, injections, 258, 264, 453

The numbers refer to the pages.

Muchæmatein, 369
Mucicarmine, 369
Mucicarminic acid, 369
Mucin, 368—370
Mucus, removal from Gastropoda, 450
Mucus cells, 368—370, 454
Mucus glands, 454
Muir, blood, 361; flagella, 483
Mullenix, inner ear, 446
Müller, C. F., silver impregnation, 217
Müller, E., salivary glands, 369
Müller, H., solution of, 42; for maceration, 273
Müller, J., paraffin imbedding, 85
Müller, W., injection, 266
Mulon, fat, 352
Munson, chloral hydrate, 238
Muscle, smooth, 341; striated, 337; nerve-endings in, 337
Musclo cells, 337
Muskens, salt solution, 237
Myelin, 404—406
Myelin stains, 407—418
Myers, celloidin, 105; serial sections, 126; Nissl's stain, 387
Myxosporidia, 479

N.

Nabias, R. de, nervous system of Pulmonata, 451; axis cylinder stain, 436
Nageotte, freezing section method, 374; myelin stain, 407, 413
Nails, 333
Nakanishi, blood, 363
Nansen, maceration, 273
Naphtha, for imbedding, 82
Naphthalin, monobromide of, 235, 244
Naphthalin red, 181
Naphthylamin brown, 384
Narcotisation, 13 *et seq.*
Nathusius, von, horn, 333
Natural injections, 269, 459, 460
Nealey, bone and teeth, 355
Neelsen and Schiefferdecker, clearing agents, 68; cedar oil, 70; origanum oil, 71; sandal-wood oil, 72
Negative impregnation, 212
Nelis, hardening ganglia, 67, 381
Nematocysts, 470
Nematoda, 278, 463; embryology, 315
Nemertina, 464
Nephelis, 18
Nephridia, of *Histriobdella*, 460
Nerve-cells, 388 *et seq.*
Nerve-endings in muscle and tendon, 337—341; in skin and others, 334—336; and *see* 447 and Methylen blue and Neurological methods
Nerve-fibres, structure, 389—406
Nervenzellenæquivalentbild, 376
Nervous centres of Reptiles, Fishes, and Amphibia, 382; of Gastropoda, 451; of Arthropods, 457; of Vermes, 460; of Actinida, 471; and *see* Neurological methods
Nervous system, *see* Neurological methods
Nesteroffsky, gold method, 222
Nettovitch, *Argulus*, 455
Nettovitch, fixation of *Argulus*, 455
Neuberger, decalcification, 282
Neubert, glycogen, 320
Neukirch, glycogen, 320
Neumayer, knife-wedges, 92; embryos of sheep, 294; reconstruction, 291; celloidin, 105
Neurokeratin, 404
Neuro-fibrils, 389—403
Neuroglia, 438—442
Neurological methods, 373; introduction and general methods, 373—385; cytological methods, 386—406; myelin stains, 407—418; myelin and axis-cylinder ditto, 418; axis-cylinder and protoplasm ditto, 419—437; retina,

The numbers refer to the pages.

inner ear, 442—447; neuroglia, 438
Neutral chromate of ammonia, 44
"Neutral" dyes, 130
Neutral red, 191; for nerve-cells, 388; for mucus cells, 360
Neutralisation, 260
Neutrophilous elements, 132
Neuville, injection, 263
New green, 194
Nicolas, gelatin imbedding, 101; osmic mixture, 32; ova of *Anguis*, 300
Nicolle, thionin, 180, 370
Nicolle and Cantacuzène, impregnation, 227
Nicotin for narcotisation, 13
Niessing, fixing liquids, 322
Nietzki, hæmatein, 152
Night blue, 483
Nigrosin, as a chromatin stain, 181; as a plasma stain, 195; for nerve-tissue, 384
Nikiforow, clearing mixture, 111
Nile blue, 353
Nilsson, alizarin stain, 198
Nissl, stain for nerve-cells, 386; hardening nerve-cells, 376, 379; Æquivalentbild, 376; Marchi's nerve stain, 415; Congo red myelin stain, 418
Nissl bodies, 386
Nitrate of copper, 57
Nitrate of silver impregnation, 214—218; generalities, 214; solutions, 215; reduction, 216; fixation, 217; marine animals, 217; injections, 262, 263; vulcanite rings for, 214; and *see* Neurological methods, Connective tissues, etc.
Nitric acid, for fixing and hardening, 39, 67, 381; for maceration, 274; for corrosion, 278; for decalcification, 280, 281, 282; for bleaching, 286
Nitrite, of amyl, 258; of silver, 428
Noack, orientation, 89
Nocht, methylen blue and red, 199
Noll, corrosion, 278; spicules, 475
Nordmann, plasma cells, 349
Normal salt solution, 236
Norris and Shakespeare, carmine and indigo-carmine, 230
Nowak, water-bath, 97; fixing mixture, 66
Nowik, corpuscles of Herbst, 334
Nuclear stains, 130; with coal-tar dyes, 169
Nuclei, *see* Cytological methods.
Nuclein, reactions, 319
Nucleoli, 194, 324
Nusbaum, serial sections, 121
Nuttall, Cooper and Robinson, Tracheata, 455

O.

Obersteiner, hardening nerve-centres, 378, 379
Obregia, serial sections, 124, 128; Golgi's impregnation, 430
Obst, nucleoli, 324
Odenius, maceration, 275
Odier, Golgi impregnation, 427
Ohlmacher, sublimate alcohol, 48, 381; section fixing, 123; mordanting with formalin, 176; safranin artefacts, 178; picro-Säurefuchsin, 188; myelin stain, 418
Oil, of aniseed, 118, 235; bergamot, 71, 81; anilin, 73, 235; cassia, 71, 235; cajeput, 72; cedar, 5, 8, 70, 81, 82, 83, 235; cinnamon, 71, 235; cloves, 8, 70, 81, 235; lemons, 235; origanum, 71; sandle-wood, 72; thyme, 72; turpentine, 72, 81, 235, 247; olive, 235; linaloa, 112
Okajima, fat, 353
Olfactive organs, 335, 447
Olive oil, refraction, 235
Olt, freezing method, 117
Opal blue, 195
Ophiuridea, 468
Oppel, liver and spleen, 370, 371
Oppitz, silver impregnation, 217

The numbers refer to the pages.

Optical differentiation, 20, 22, 37

Orange G, 184, 187, 189

Orcein, Israel's method, 229; Unna's, 345, 347

Orcein-Wasserblau mixture, 334

Orcholla, 229

Orientation, in paraffin, 86, 87; in celloidin, 106; of blocks, 88; of ova, 297

Origanum oil, 71

Orr, Marchi's nerve-stain, 415, 417

Orseille, 229

Orth, "Formol-Müller," 66, 380; lithium carmine, 147

Orthoptera, embryology, 611

Osmic acid, generalities, 28, 31; how to keep, 28; regeneration of, 29; fixation with, 29; after-treatment, 30; characters of the fixation, 31; blackening of fat, 352; mixtures, 31, 35 *et seq.*; sublimate mixtures, 50; picric mixtures, 59; stains with pyrogallol, pyroligneous acid or tannin, 226; for hardening, 381; stains for medullated nerve, 416, 417; for maceration, 273

Osmic-bichromic mixtures, 37, 38

Osmio-sublimate mixtures, 50

Osmium hæmatoxylin, 168

Osmium, *see* Osmic acid.

Osmium-carmine, 466

Osmium chloride, 52

Osmosis, to avoid, 3

Östergren, ether-water, 15; Nemertians, 464; *Dendrocœlum*, 467; *Synapta*, 467

Ostracoda, 454

Otocyst of *Mysis*, 456

Ova, *see* Embryological methods.

Ovens, 85

Overton, bleaching, 30, 33; fixing with iodine, 53; fixing Algæ, 44

Oviatt and Sargent, injecting, 258

Oxalic acid, for maceration, 275

Oxychloride of ruthenium, 227

Oxygenated water, for narcotisation, 18; for bleaching, 30, 285

P.

Pacaut, picro-sublimate, 50

Pacini, preservative liquids, 239

Pal, nerve-stain, 411; Golgi's sublimate method, 433

Paladino, nerve-stain, 418

Palladium chloride, for fixing, 52; staining, 227, 418; decalcifying, 280

Palladium chloride, 52, 227, 280, 418

Pancreas, 370

Pancreatin digestion fluid, 276, 277

Paneth, goblet-cells, 369

Panoptic stain, 363

Pansch, starch injection, 268

Paper cell mounting method, 250

Paper trays and thimbles, 77, 78

Papillæ foliatæ, 335

Pappenheim, plasma cells, 349, 351; panoptic stain, 363; blood-plates, 367; chemistry of staining, 129; neutral dyes, 132; methyl green and pyronin, 183, 350; hæmopoietic tissues, 366; sublimate fixative, 48

Paracarmine, 149

Paraffin, for preserving material, 5; solvents of, 81, 82; imbedding in, 81—87, orienting in, 86; cutting, 88—96; ribbons, 96; coating blocks of, 97; electrification of, 97; masses recommended, 98; mounting sections, 98; ascertainment of melting point, 99; overheated, 99; cement, 250, 252

Paraffinum liquidum, 235

Paravicini, neurofibrils, 402

Paris violet, 172

Parker, dehydration, 4; methylen blue, 208; turpentine cement, 252; bleaching, 286; eyes of Arthropods, 457

Parker and Floyd, formol for brain, 377

Parma blue, 195

Parolein, 248

Parthenogenesis, 287

The numbers refer to the pages.

Partsch, cochineal, 143; decalcification, 282

Passarge and Krösing, elastic tissue, 348

Patellani, hæmatoxylin, 167

Paton, embryological method, 296 neurofibrils, 400

Patten, orientation in paraffin, 86; embryology of Blattida, 311; eyes of Lamellibranchs, 452 maceration of Mullusca, 453

Patterson, ova of *Columba*, 297

Pauropoda, 455

Pavlow, celloidin, 104; Pal's nerve-stain, 412

Pedicellariæ, 468

Peabody, corrosion, 278

Peirce, labelling slides, 484

Pekelharing, glycogen, 320

Pelagic ova, 305

Pelletierin, 450

Pensa, reconstruction, 290; mitochondria, 330

Pentacrinus, 470

Peppler, flagella, 482

Pepsin digestion fluids, 276

Perchloride of iron, for fixing, 53; for staining, 227

Peremeschko,larvæ of Amphibia,318

Perényi, chromo-nitric acid, 39, 300

Pérez, pupæ of Diptera, 310

Periplaneta, embryology, 309, 311

Permanganate of potash, for bleaching, 30, 285; for mordanting, 176; for maceration, 273

Peroxide of hydrogen, for narcotisation, 18; for bleaching, 30, 285

Peroxide of magnesium, 285

Peroxide of sodium, 285

Perrier, *Lumbricus*, 458

Peter, orienting, 87; iron cochineal, 146; reconstruction, 291

Petroleum-ether, 81, 82

Petrone, medullated nerve, 405

Petrunkewitsch, fixing liquid, 49; ova of Hymenoptera, 611

Pfeiffer von Wellheim, iron-carmine, 145

Pfister, hardening nerve-centres, 376

Pfitzer, picro-nigrosin, 195

Pfitzner, safranin, 177; Protozoa, 477

Phalangida, embryology, 312

Phénicienne, La, 172

Phenol sublimate, 48

Phenylen brown, 172

Philippson, epidermis, 331

Phloroglucin, 283

Phloxin, 192

Phospho-molybdic hæmatoxylin, 167

Phosphoric acid, 280, 283

Phosphorus, detection of, 320

Phospho-tungstic hæmatoxylin, 167

Photoxylin for imbedding, 102, 103; for injection, 269

Physa, ova, 309

Physiological salt solution, 236

Pianese, formic acid carmine, 145; methylen blue and eosin, 193; carmine and picro-nigrosin, 231; Säurefuchsin, 187

Piazza, ripening hæmatoxylin, 153

Picrate of ammonia, 130

Picrate of silver, 216

Picric acid, 57; washing out, 57; as a plasma stain, 187; for decalcification, 280, 283

Picric alcohol, 58, 275

Picro-acetic acid, 58

Picro-alum-carmine, 145

Picro-carmine, 147, 148

Picro-chromic acid, 59

Picro-formol, 65

Picro-hydrochloric acid, 59, 283

Picro-indigo-carmine, 230

Picro-magnesia-carmine, 148

Picro-nigrosin, 195, 231, 344, 384

Picro-nitric acid, 58, 283

Picro-nitro-chromic acid, 59

Picro-osmic acid, 59

Picro-platinic mixtures, 59, 66, 289

Picro-Säurefuchsin, 188, 344; with hæmatoxylin, 232

Picro-sublimate mixtures, 49

Picro-sulphuric acid, 58

The numbers refer to the pages.

Pictet, examination liquid, 237
Pigment, removal of, 456, 457; and *see* Bleaching.
Pigment spots, artificial, 43, 381
Pintner, Tæniæ, 465; osmic acid, 28
Pisces, embryology, 303
Pisenti, alum-carmine, 143
Pitfield, flagella, 482
Pizon, gemmation of Ascidians, 306
Planaria, 466, 467; embryology, 314
Planorbis, embryology, 308
Plasma cells, 349—351
Plasma fibrils, 332
Plasma stains, defined, 130; the coal-tar, 182; cytological, 325—330
Plastic reconstruction of sections, 290
Platino-aceto-osmic acid, 38
Platino-sublimate mixtures, 52
Platinum chloride, 51; mixtures, 38; 40, 52, 289, 322
Platner, medullated nerve, 53, 405; Kernschwarz, 228
Plečnic, imbedding, 82
Pleschko, methylen blue, 208
Plessis, Du, *see* Du Plessis.
Pluteus, 150, 469
Podwyssozki, fixing mixture, 36; safranin, 178
Poisoning, 17 *et seq.*
Polaillon, iron impregnation, 227
Polarisation, for myelin, 417
Policard, Trypanosomes, 480
Police, hæmatoxylin, 167
Politzer, inner ear, 445
Polumordwinow, stain for nerve-cells, 388
Polychœrus ova, 314
Polychrome toluidin blue, 211
Polychrome mythylen blue, 199
Polzam, soap imbedding, 100
Popow, neuroglia, 442
Porifera, 474, 475
Positive impregnation, 212
Poso, paraffin imbedding, 85, 383
Post-impregnation, 219, 224
Potash, for maceration, 272; for corrosion, 278; acetate of, *see* Acetate; bichromate of, *see* Bichromate; hypochlorite of, *see* Hypochlorite; permanganate of, *see* Permanganate.
Potassium, detection of, 321
Potassium bichromate, *see* Bichromate.
Potassium ferricyanide, for bleaching, 31
Potassium permanganate, for bleaching, 30, 285; as a mordant, 176; for maceration, 273
Pötter, myelin stain, 412
Pouchet, bleaching, 285
Pranter, imbedding, 82; elastic tissue, 347, 348
Pre-impregnation, 219, 220
Prenant, safranin, 178, 194; cochlea, 446; ergastoplasm, 327; microchemistry, 321; mitochondria, 329
Preservation of material, 4
Preservative media, *see* Examination and Preservation.
Preyer, star-fishes, 14
Prickle-cells, 331
Primerose, 192
Pringle, vacuum imbedding, 86
Pritchard, chromic acid mixture, 34; reducing liquid, 222; cochlea, 447
Progressive staining, 138, 169—173
Protargol, 216
Protozoa, 474—483
Prowazek, neutral red, 192
Prudden, hæmatoxylin, 163
Prussian blue, impregnation, 227; injections, 259, 261, 262, 265, 266; soluble, 261, 262
Przesmycki, Protozoa, 477, 479
Purcell, eyes of Phalangida, 457; ova of *Atta*, 312
Purpurin, 229
Pusateri, silver stain, 397
Pyridin, 62
Pyrogallate of iron stain, 227

Pyrogallate of osmium stain, 226
Pyroligneous acid, 280
Pyroligneous acid carmine, 145
Pyroligneous acid hæmatoxylin, 164
Pyronin, 183, 190, 350
Pyrosin, 192
Pyrosoma, 462
Pyroxylin, 105

Q.

Quervain, de, fixation of nervous tissue, 374
Quieting Infusoria, etc., 461, 476
Quinoleïn blue, 195

R.

Raadt, blood parasites, 366
Rabbit, embryology, 291—294
Rabl, chromo-formic acid, 35; picro-sublimate, 49; platinum-sublimate, 52; platinum chloride, 52, 289; paraffin sections, 96, 98; cochineal, 144; hæmatoxylin and safranin, 232; embryological methods, 289, 302, 304; horny tissues, 333; crystalline, 336; section fixing, 123
Rabl-Rückhard, embryology of Salmonidæ, 304
Racovitza, mucus glands, 454
Raffaele, pelagic ova, 305
Raja, 305, 340
Ramón y Cajal, picro-Säurefuchsin, 188; picro-indigo-carmine, 230, 344; nerve-endings in muscle, 338; stain for nerve-cells, 388; Golgi impregnation, 426, 428, 429; methylen blue, 435; retina, 444; connective tissue, 344; neurofibrils, 390—398; myelin stain, 416; gold impregnation, 436
Rana, embryology, 302
Randolph, chloreton, 15
Ransom, Cestodes, 465
Ranvier, osmic acid, 31; chromic acid, 34; one-third alcohol, 62; absolute alcohol, 62; ammonia-carmine, 146; picro-carmine, 147; quinoleïn blue, 195; purpurin, 229; impregnation, secondary, 213; impregnation, with silver, 214, 215; with gold and formic acid, 221; with gold and lemon-juice, 221; after blackening of gold, 225; iodised serum, 237; injections, carmine, 259; injections, Prussian blue, 261, 266; impregnation injections, 263; maceration, 271, 273, 275; decalcification, 282; tactile corpuscles, 334; cornea, 335, 336; nerve and muscle, 338; plasma fibrils, 333; corpuscles of Golgi, 340; bladder of frog, 343; medullated nerve, 416; electric organs, 339; retina, 443, 445; areolar tissue, 345; eleidin, 333; clasmatocytes, 351; bone, 353; cochlea, 446; glands, 371
Rawitz, picro-nitro-chromic acid, 59; picro-nitro-osmic acid, 59; carmalum, 144; inversion stains, 197; artificial alizarin, 197; bleaching, 286; mucicarminic acid, 369; eyes of Lamellibranchs, 452; phospho-tungstic fixative, 39; cochineal, 144; glychæmalum, 163; cœrulein, 194; osmium stain, 226; hardening brain, 380; stains for nerve centres, 385
Reagents, 11
Recklinghausen, silver impregnation, 215, 216
Reconstruction, from sections, 289; and *see* Orientation.
Redding, gold impregnations, 225
Reddingius, nucleoli, 325
Redenbaugh, narcotisation, 17
Refraction, indices of, 235; of protoplasm, 20, 234
Regaud, silver impregnation; 216; hæmatoxylin stain, 232; mitochondria, 328; myelin stain, 407

The numbers refer to the pages.

Regaud and Dubreuil, protargol, 216
Regressive staining, 139; with tar colours, general directions, 173—176, 180
Rehm, benzin colophonium, 246; stains for nerve-cells, 387
Reichenbach, ova of *Astacus*, 313
Reichert, microtome, 10
Reinhold-Giltay, microtome, 11
Reinke, gentian and orange stain, 190; lysol, 276; horny tissue, 333
Rejsek, corrosion, 277
Rejuvenation of Golgi material, 426
Remak, hardening ova, 303
Remounting, 7
Renaut, hæmatoxylin and eosin, 232; silver staining, 216; cornea, 336; mitochondria, 330
Reptilia, embryology, 299; nervous centres, 382
Resegotti, staining by substitution, 175; safranin, 178
Resins and balsams, 244
Resorcin, imbedding method, 118
Resorcin fuchsin, 348
Re-staining old mounts, 7
Retina, 19, 442—445
Retterer, embryology of rabbit, 293; bichromate sublimate, 51; tendon, 340; smooth muscle, 342
Retterer and Lelièvre, muscle and tendon, 340
Retterer and Zellner, natural injections, 269
Retzius, methylen blue, 205, 207
Rezzonico, medullated nerve, 404
Rhabdites, 466
Rhopalæa, 17
Rhumbler, paraffin imbedding, 79; methyl green and eosin, 193
Ribbert, phospho-molybdic hæmatoxylin, 167
Ribbon, section-cutting, 92, 96
Richards, narcotisation, 16
Rieder, fat, 352
Ringer's solution, 236
Ringing wet mounts, 249, 250, 252
Ripart and Petit's liquid, 56, 318
Ripening of hæmatoxylin, 152—154
Ritter, Ascidians, 306
Robert, *Aplysia*, 450
Robertson, imbedding method, 115; nerve-stains, 414, 417; platinum, impregnation, 434
Robin, injections, 258, 259, 262; natural injections, 269
Robinski, silver impregnation, 215; crystalline, 336
Rochon-Duvigneaud, retina, 443
Rodin, salt solutions, 237
Roessle and Yoshida, lymphatic glands, 371
Rollett, freezing method, 118; cornea, 273, 336
Romanowsky, stain, 364, 480
Roosevelt, pyrogallate of iron, 227
Röse, bone, 354
Rose B à l'eau, 192
Rose de naphthaline, 181
Rosein, 181
Rosenstadt, plasma fibrils, 332; eyes of Decapods, 458
Rosenthal, fat, 352
Rosin, "neutral" dyes, 131, 193; neutral red, 191, 389
Ross, blood, 363
Rossi, nerve stain, 414; blood, 360; flagella, 482
Rossolimow and Busch, nerve-stain, 417
Rotatoria, 461, 472; embryology, 314
Roth aus Methylenblau, 199
Röthig, Kresofuchsin, 181; stains for nervous tissue, 385
Rouget, methylen blue, 205; silver impregnation, 216, 217
Rousseau, decalcification, 279, 356; desilicification, 284; sponges, 474
Rousselet, cements, 250, 251; aqueous mounts, 250; Rotatoria, 461

The numbers refer to the pages.

Rubaschkin, neuroglia, 440; serial sections, 125
Rubin, basic, 181; "acid," Rubin S, 183
Rubinstein, blood, 359
Ruffini, corpuscles of Golgi, 341
Ruppricht, serial sections, 124, 125
Ruprecht, bone, 355
Russo, *Ophiothrix*, 469
Ruthenium, impregnation, 227; red, 227
Ruzicka, nerve-cells, 325; orcein, 229; nucleoli, 325
Ryder, double imbedding, 114

S.

Sabussow, double imbedding, 114
Sacerdotti, Golgi's impregnation, 426
Saefftigen, *Echinorhyncus*, 462
Saffrosin, 192
Safranelin, 348
Safranin, 176–178; for elastic tissue, 347; for bone, 356; for mucus cells, 368; for nerves, 418; with indigo-carmine or nigrosin, 195; with Wasserblau, 196; with Lichtgrün or Säure-violett, 194; with Kernschwarz, 228; with hæmatoxylin, 232
Sahli, balsam, 245; hardening nerve-centres, 376, 378; myelin and axis-cylinder stains, 418
Sala, neuroceratin, 405
Salamandra, embryology, 302
Salicylic acid, 56
Saling, ova of *Tenebrio*, 312; larvæ of *Tenebrio*, 456
Salivary glands, 369
Salkind, thymus, 372
Salmonidæ, embryology, 304
Salpa, 448
Salt solution, 236; for maceration, 271, 272
Salts, metallic, action of light on, 213
Samassa, Golgi's impregnation, 430; Ctenophora, 474
Samter, paraffin imbedding, 79, 87
Sánchez, methylen blue, 206; neuro-fibrils, 397
Sand, neuro-fibrils, 400; neuroglia, 440
Sandal-wood oil, 72
Sandarac for mounting, 247, 248
Sanders, Cox's sublimate impregnation, 433
Sankey, anilin blue-black, 384
Sanzo, apparatus for fixing, 289
Saragnone, intracellular network, 404
Sarcolemma, 337
Sata, fat, 352
Sattler, silver impregnation, 217
Sauer, kidney, 372
Säurefuchsin, 130, 183; and orange, 184, 232; and hæmatoxylin, 232
Säurerubin, 183
Säureviolett, 194
Saville Kent, Infusoria, 53
Savini, picro-Säurefuchsin, 189, 344; elastic tissue, 347; Nissl's stain, 388
Sazepin, antennæ, 456
Scala, Protozoa, 478
Scarpatetti, axis cylinder- and cell-stain, 385
Schäfer, muscle-cells, 337
Schaffer, decalcification, 280, 281, 283; reconstruction, 290; retina, 445; bone, 353, 354, 356; dehydration, 4; paraffin blocks, 89; lime salts, 320; connective tissue, 344; plasma cells, 351
Schällibaum, collodion, 123
Schanze, microtome, 9
Schaper, reconstruction, 291
Scharlach R., 352
Schaudinn, Protozoa, 478, 479
Schenk, acetate of uranium, 57
Schewiakoff, Protozoa, 478
Schiefferdecker, clearing agents, 68, 71; serial sections, 119; celloidin imbedding, 103; injections, 268; methyl mixture, 275; digestion, 277; retina, 445; car-

The numbers refer to the pages.

tilage, 357; muscle, 337; microtome knives, 90; Weigert's myelin stain, 409
Schlemmer, silver bath, 398
Schmaus, anilin blue-black, 384; nerve-stain, 384
Schmidt, F., embryology of Pulmonata, 308
Schmidt, G., methylen blue, 209
Schmorl, bone, 357; serial sections, 122
Schneider, Aimè, injection of Arthropods, 458
Schneider, Anton, aceto-carmine, 145
Schneider, R., iron, 320
Schoebel, washing apparatus, 4
Scholz, acetone, 63
Schönemann, serial sections, 124; reconstruction, 291
Schonlein, *Aplysia*, 450
Schreiber, Golgi's impregnation,428
Schridde, decalcification, 282; fixation, 288; plasma-cells, 351; blood, 361
Schrötter, stains for nervous tissue, 385, 414
Schuberg, dahlia, 180; Romanowsky stain, 366, 480; cilia of Infusoria, 475
Schuberg and Schroeder, maceration, 275
Schultz, smooth muscle, 342
Schultze, F. E., palladium chloride, 52, 227; section-stretcher, 94; dehydration, 4
Schultze, Max, iodised serum, 237; acetate of potash, 30, 238; retina, 445
Schultze, O., ova of Amphibia, 302; hæmatoxylin, 166, 168; muscle and tendon, 340
Schumacher, elastic tissue, 348; spleen, 371
Schürmayer, Infusoria, 476
Schütz, neurofibrils, 399
Schwalbe, impregnation, 213; cochlea, 445
Schwarze, Cercariæ, 466
Schydlowski, paraffin imbedding, 79
Sclavo, flagella, 483
Scott, F. H., blood, 362; phosphorus, 320
Scott, S., inner ear, 447
Scott and Osborn, embryology of *Triton*, 301
Scyphistomes, 473
Sealing-wax varnish, 253
Secretion granules, 327
Sections, collodionisation, 95, 383; cutting paraffin, 93; ribbons of, 96; rolling of, 93—95; flattening, 97; mounting, 119 *et seq.*; reconstruction from, 289
Section-flattening, 97
Section-grinding, 115
Section-stretchers, 94
Section-stretching, 93—95
Seeliger, *Antedon*, 470
Sehrwald, Golgi's impregnation, 429, 430
Seidenmann, methylen blue, 206
Seiler, carmine and indigo-carmine, 229; alcohol balsam, 246; decalcification, 283
Selachii, embryology, 305; blood, 237
Selenka, imbedding apparatus, 79, 88; embryology, 294
Seligmann, methods for the eye, 442
Serial section mounting, 119 *et seq.*; paraffin sections, 119 *et seq.*; watery sections, 125; celloidin sections, 125
Serum, iodised, 237, 238; maceration in, 271
Severeanu, injection, 268
Shearer, nephridia, 460
Shell, 452
Shellac, for imbedding, 115, 117; varnish, 253; injection masses, 268
Sida, ova, 313
"Siebdosen," 4

The numbers refer to the pages.

SIEBENMANN, labyrinth, 447
Sieve-dishes, 4
SILBERMANN and OZOROWITZ, glycogen, 320
Silver nitrate, *see* Nitrate of silver.
Silver, other salts, 216
Siphon, Ewald's capillary, 4
Siphonophora, 473
Siredon, embryology, 301
Sixth sense, 335
SJÖBRING, formol, 64
SJÖVALL, intracellular network, 404
Skin, 331 *et seq.*
SKROBANSKY, anilin blue, 196
Slides, cleaning, 121, 484
SMIRNOW, tactile corpuscles, 334; epidermis of *Lumbricus,* 424
SMITH, G., eyes of Gastropoda, 451
SMITH, HOPEWELL, odontoblasts, 355
SMITH, LORRAIN, fat, 353
SMITH, S., serial sections, 122
Smooth muscle, 341
SNESSAREW, connective tissue, 346
Soap imbedding, 100, 465
SOBOTTA, imbedding ova, 289; ova of mouse, 294; of rabbit, 294; of Salmonidæ, 304; of *Amphioxus,* 305
Soda, for maceration, 272; for corrosion, 278; for bleaching, 286; hypochlorite of, *see* Hypochlorite.
Soda carmine, 146, 384
Sodium, iodate, 32; chloride, 236; peroxide, 285
Solferino, 181
SOLGER, bleaching, 285; salivary glands, 369; sarcolemma, 337
Solid green, 194
SOLLAS, freezing mass, 118
SONNENBRODT, bichromate of calcium liquid, 44; ovary of fowl, 44
SOULIER, maceration, 272
SOUZA, pyridin, 62
SPEE, prepared paraffin, 99
Sphærozoa, 479
Spicules of sponges, 278, 474
SPIELMEYER, myelin stain, 407
"Spiegelfärbung," 326
Spinal cord, *see* Neurological methods.
Spines of Echinoidea, 468
Spirit-proof cement, 251
Spirit blue, 195
Spleen, 371
Sponges, 278, 474, 475
Sporozoa, 478, 479
SPULER, sublimate, 48, 51, 66; formol mixture, 66; iron cochineal, 146
SQUIRE, benzol, toluol, xylol, 73; origanum oil, 72; bluing hæmatoxylin stains, 160; Kleinenberg's hæmatoxylin, 165; methyl green, 172; Säurefuchsin, 184; Ehrlich-Biondi stain, 185; salts of gold, 219; hæmatoxylin and Säurefuchsin, 232; glycerin jelly, 242; decalcification, 282, 283
SSOBOLEW, honing knives, 91
"Stabilit," 110
STAHL, immobilisation of Infusoria, 476
Staining, in bulk and on the slide, 7; tubes for, 7; reagents for, 11; generalities on, 129—142; with carmine, 141; with hæmatoxylin, 152; with tar colours, 169; practice of, 138; old mounts, 7; theories of, 129; progressive, 138, 169; regressive, 139, 173; substantive and adjective, 133; by substitution, 175; *intra vitam,* 136, 201, 203
Stains, combined, 228 *et seq.*; combined, with carmine, 229; combined, with hæmatein, 231
Stains, kinds of, 129; specific, 129; nuclear, 130, 324; plasmatic, 130; inverted, 135; choice of, 140; cytological, 324—330; metallic, 212—227
STAPPERS, fixation of Sympoda, 455
Starch, injection, 268
STARKE, osmicated fat, 352
STATKEWITSCH, Protozoa, 476

The numbers refer to the pages.

Statoblasts, 306

Stauffacher, embryology of *Cyclas*, 309

Stein, temporal bone, 279, 446

Steinach, sieve-dishes, 4

Stempell, Sporozoa, 479

Stepanow, celloidin, 114; double imbedding, 114; freezing method, 118

Stephens, stain for flagella, 483

Stephenson's high refractive medium, 243

Sterling, double imbedding, 114

Stirling, maceration, 272

Stoelzner, sublimate solution, 48; lime salts, 320; myelin stain, 407

Stöhr, eosin, 231

Storax, *see* Styrax.

Storch, Weigert's neuroglia stain, 440

Stovaine, for narcotisation, 462

Stoves, 85

Strahuber, axis cylinder stain, 437

Strasser, section-stretcher, 94; collodion-paper method, 124, 128; gelatin method, 125; reconstruction, 291; imbedding nervous system, 383

Strecker, hardening and staining, 378

Streeter, myelin stain, 414

Stricht, van der, bergamot oil and fatty granules, 71; decalcification, 283; ova of Turbellaria, 314

Stricker, gum imbedding, 115

Stroebe, myelin stain, 418

Strong, fixing nerve-centres, 382; Golgi impregnation, 427; myelin stain, 415, 417; fixing mixture, 67

Stropeni, plasma cells, 350

Studnicka, connective tissue, 346

Stylaria, 18

Styrax, 248

Sublamin, 51

Sublimate, corrosive, generalities, 45; fixation with, 45 *et seq.*; acetic solutions, 45; various mixtures, 47, 48, 50, 66; alcoholic, 48; preservative liquids, 239; hardening, 381

Substantive staining, 133

Substitution in staining, 175

Subtriessig, 56

Suchannek. sieve-dishes, 4; bergamot oil, 71; anilin oil, 73; serial sections, 119; Venice turpentine, 246

Sudan III, 352

Sulima, chloreton, 15

Sulphate of copper, for fixing, 43, 303, 473; for staining, 227; for impregnation, 437

Sulphate of chromium, 380

Sulphate of iron, 156

Sulphate of magnesia, narcotisation, 17

Sulphide of carbon, 82, 235

Sulphides for impregnation, 227

Sulphindigotate of soda, 229

Sulphocyanides, 272

Sulphorhodamin, 138

Sulphur, refractive index, 235

Sulphuric acid, for maceration, 275

Sulphurous acid, for bleaching osmic material, 31; for bichromate ditto, 42, 285; for fixing, 44; for decalcification, 282

Sumita, iron, 320

Summers, ether method, 125

Sunning metallic salts, 213

Suschkin, embryos of birds, 297

Sussdorf, mucin, 368

Susuki, dehydration, 4

Syrup, mounting media, 238; for freezing, 117, 118

Szütz, alizarin stain, 198; gold method, 225; mito-chondria, 330

T.

Tachiol, 397

Tactile corpuscles, 334

Tænia, 465; ova, 314

Taenzer, elastic tissue, 347

The numbers refer to the pages.

Tafani, inner ear, 447
Taguchi, injection, 267
"Tal," Golgi's sublimate method, 433
Tandler, injection, 264
Tannin, examination medium, 239; for demonstrating cilia, 477
Tar colours, 169 *et seq.*
Tardigrada, 458
Tartuferi, cornea, 336; retina, 444
Teeth, 354—356
Tegumentary organs, 331—336
Teichmann, white injection, 262; linseed oil injection, 268
Teleostea, embryology, 303
Teljatnik, myelin stain, 417
Tellyesnicky, nitric acid, 39; acetic bichromate, 42; formol mixture, 65; toning bath, 392
Telsons, 456
Tendon, 340 *et seq.*
Tenebrio, ova, 312; larvæ, 456
Terpinol, 72; index, 235
Test-cells of Ascidians, 306
Tetrachloride of carbon, 81, 82
Thanhoffer, silver nitrate, 217
Thate, microtome, 10
Théohari, granules, 323
Theridium, ova, 312
Thiersch, indigo-carmine, 229; carmine injection, 261; Prussian blue injection, 262; green and yellow injection, 262
Thimbles, paper, 78
Thin, retina, 271
Thionin, 179; for impregnation, 210; for nerve-cells, 388; for mucus-cells, 368
Thiophen green, 194
Thoma, microtome, 9; decalcification, 281; corrosion, 277; injection, 260
Thomé, Ehrlich-Biondi stain, 185
Thread-cells, 470
Thus, gum, 248
Thyme oil, 72
Thymus, 372
Tigroid bodies, 386—389, 398
Timofejew, hardening nerve-tissue, 381; methylen blue, 210; liver, 371
Tin hæmatoxylin, 168
Tintinnodea, 478
Tirmann, iron, 320
Tischatkin, dehydration, 4
Tizzoni, alum-carmine, 143
Tobacco narcotisation, 13, 17, 317
Toison, blood, 362
Tolu balsam, 235; cement, 253
Toluen, *see* Toluol.
Toluidin blue, chromatin stain, 180; for impregnation, 210; for nerve-cells, 388; for neuro-fibrils, 401 for myelin, 414
Toluol, for clearing, 73; for imbedding, 81; for preserving, 5
Tomaselli, neuro-fibrils, 402
Tongue, of frog, 335; of rabbit, 335
Toning baths for silver stains, 392, 393, 397, 398, 399, 431
Tonkoff, bleu de Lyon, 196
Tornier, hæmatoxylin and sublimate, 166
Torpedo, 339
Tortoise, embryology, 299
Tourneux and Herrmann, silver impregnation, 216
Tower, Cestodes, 465
Tozer, Rotifers, 462
Tracheæ, 456
Tracheata, 455
Trambusti, Ehrlich-Biondi stain, 186
Trays for imbedding, 77
Trematodes, 465; embryology, 315
Trenkmann, stain for flagella, 483
Trezebinski, ganglion cells, 381
Triacid mixture, 187
Trichinæ, 463
Trichloracetic acid, 56, 282
Trichlorlactic acid, 56
Trichohyalin, 333
Triton, embryology, 301
Trypanosomes, 480
Trypsin digestion, 276, 277
Tschernischeff, colloxylin, 103, 114

The numbers refer to the pages.

TSCHERNYSCHEW and KARUSIN, nerve-stain, 412

TSCHISCH, liquid of Erlicki, 43

TUBBY, celloidin, 104

Tubes for staining on slide, 7

TULLBERG, narcotisation, 17

Tunicata, 448; embryology, 306; gemmation, 306

Turbellaria, 466; embryology, 306, 314

Turpentine, cement, 252

Turpentine, oil of, for clearing, 72; for imbedding, 81; for mounting, 247

Turpentine, Venice, 246; cement, 252

TWORT, stain for blood-parasites, 480

U.

UEXKULL, asphyxiation, 18

UNDERWOOD, teeth, 356

Unio, ova and glochidia, 309

UNNA, osmic mixture, 32; washing out chromic objects, 33; ripening hæmatoxylin, 152; hæmatoxylin solution, 162, 164; keratohyalin, 333; plasma fibrils, 332; smooth muscle, 341; connective tissue, 344, 345; plasma cells and Mastzellen, 349, 350; elastic tissue, 347; fibrin, 368; mucin, 368; celloidin, 103; prickle cells, 332; glycerin ether, 349; oil of Gaultheria, 72; carbol-pyronin, 183; polychrome methylen blue, 199; digestion, 320; Wasserblau and orcein, 332, 334, 341, 344; tyrosin, 334; orcein for elastic tissue. 347

UNNA and GOLODETZ, iron stain, 227; keratohyalin, 333

UPSON, impregnation methods, 384, 436

Uranium, acetate, 57

USSOW, ova of Cephalopoda, 307

V.

Vacuum imbedding, 85, 86

VALLE, DELLA, Amphipoda, 313

VAN BENEDEN, *see* BENEDEN; and for all names with the prefix VAN *see* the patronymics.

Vanadium chloride stain, 435

Vanadium hæmatoxylin, 167

Varnish, negative, for mounting, 248

Varnishes, 249 *et seq.*

Vaso-dilators for injections, 257, 453

VASSALE, Weigert's nerve-stain, 409; Marchi's nerve stain, 415

VASSALE and DONAGGIO, Golgi's impregnation, 428

VASTARINI-CRESI, medullated nerve, 417; section cutting, 94; glycogen, 320; cartilage, 357

VEJAS, nerve-tissue, 384

VEJDOVSKY, *Gordius*, 463

VENDEROVIC, Marchi's nerve stain, 416

Venice turpentine, for mounting, 246; cement, 252

Ventral cord, of Arthropods, 457

VERATTI, Golgi impregnation, 403

VERHOEFF, elastic tissue, 348

Veretillum, 13

Véridine, 170

Vermes, 458 *et seq.*; embryology, 314—316

Vert en cristaux, v. lumière, v. d'Eusèbe, v. d'alcali, 170

VERWORN, *Cristatella*, 15

Vesuvin, 172

VIALLANES, collodion imbedding, 107; gold method, 221; eyes of *Palinurus*, 458

VIALLETON, ova of fowl, 298; of Cephalopods, 307

Victoria blue, 180

Victoria green, 194

VIGNAL, osmic acid, 31

VILLE, carmine injections, 260

Violet B, 196; of Lauth, 179

VIRCHOW, action of light on chromic objects, 33

Visibility, index of, 234, 245

Vivante, bone, 356

Vogt and Yung, Annelids, 458; Gephyrea, 461; Cestodes, 464; Holothurids, 467

Volk, peroxide of hydrogen, 18; Rotifers, 461

Vom Rath, picro-sublimate mixtures, 50; osmio-sublimate, 50; picro-osmic acid, 59; picro-platinic mixtures, 59

Von Ebner, *see* Ebner; and for all names with the prefix von *see* the respective patronymics.

Vorticellidæ, 16

Vosmaer, section grinding, 116; reconstruction, 290

Vosmaer and Pekelharing, sponges, 474

Vosseler, Venice turpentine, 246; wax feet, 270; cement, 252

W.

Waddington, narcotisation, 14; fixing Infusoria, 44; demonstrating cilia, 477

Waite, ova of *Homarus*, 313

Waldeyer, decalcification, 280; cochlea, 447

Walsem, van, knife warmer, 93; paraffin, 99; nerve-stain, 414

Ward, asphyxiation, 18; *Sipunculus*, 461

Ward's brown cement, 250

Washburn, embryology of Gastropoda, 308

Washing out fixing liquids, 2, 25, 32

Wasielewsky, Sporozoa, 479

Wasserblau, 196, 345, 341

Wassermann, iron, 320

Watasé, ova of Cephalopoda, 307

Water, fresh or warm, for killing, 18; refractive index, 235; sea-water, do., 235; as an examination medium, 236; method for serial sections, 119; test for, 62

Water-baths, 85

Water-blue, 196

Wax feet, 270

Webb, dextrin freezing mass, 118

Weber, A., serial sections, 125

Weber, E., Rotatoria, 461; Echinoidea, 468; Siphonophora, 473; Asteroidea, 468; Holothurids, 468

Webster, naphtha for imbedding, 82

Wedl, orchella, 229

Weidenreich, eleidin, 333; blood, 360; spleen, 371

Weigert, serial sections, 127; clearing celloidin, 111; Bismarck brown, 172; picro-Säurefuchsin, 189, 233; varnish, for mounting, 248; hardening nerve centres, 375, 377; stain for nuclear figures, 159; myelin stains, 408—414, 415; Golgi's impregnation, 425; neuroglia stain, 438; elastic tissue, 348; fibrin stain, 367; iron hæmatoxylin, 159, 233

Weil, section-grinding, 117; teeth, 355

Weiss, P., address, 11

Welcke, flagella, 483

Wellheim, Pfeiffer von, iron-carmine, 145

Wermel, blood, 362

Werner, smooth muscle, 342

Wester, chitin, 456

Weysse, embryology of *Sus*, 294

Whartonian jelly, 368

Wheeler, embryology of Blattida, 311

Whetting knives, 91

White, bone and teeth, 354

White of egg, freezing method, 118; section fixatives, 122, 123; examination media, 237, 239; injection mass, 267; refractive index, 235

Whiting, spleen, 371

Whitman, chromo-platinic mixture, 40; ova of Amphibia, 300, 302; pelagic ova, 305; Hirudinea, 460

Wickersheimer's fluid, 239

The numbers refer to the pages.

WIDAKOWICH, embryology of mouse, 295
WIDMANN, eyes of Arachnida, 458
WIJHE, VAN, ammonia-carmine, 146; picro-carmine, 147; cartilaginous skeletons, 358
WILHELMI, Triclads, 67, 467; double imbedding, 114; picro-Säure-fuchsin, 189
WILL, embryological methods, 299
WILLEBRAND, blood, 363
WIMMER, neuroglia, 440
WINIWARTER, embryological methods, 295
WINIWARTER and SAINMONT, orange method, 189
WILSON, Alcyonaria, 472; orientation, 87; imbedding box, 79
WINTERSTEINER, serial sections, 128
WISTINGHAUSEN, VON, hæmatein stains, 160
WITT, DE, elastic tissue, 348
WITTMAACK, myelin stain, 417; inner ear, 446
WOLFF, ELISE, staining bichromate material, 42; thionin stains, 180; fibrin, 368; elastic tissue, 347; orcein, 229
WOLFF, M., freezing, 117
WOLFF, W., bladder of frog, 342
WOLFRUM, elastic tissue, 348
Wollschwartz, 481
WOLTERS, hæmatoxylin nerve-stain, 412; vanadium nerve-stain, 435; cartilage and bone, 357
WOODLAND, Holothurids, 468; larvæ of Echinoderms, 470
WOODWORTH, orienting in paraffin, 87; reconstruction, 290
WRIGHT, blood-cells, 367; blood-plates, 367
WUNDERER, nerve-endings, 338
WYNN, nerve-stain, 412

X.

Xylol, index of, 235; for clearing paraffin sections, 73; celloidin sections, 111; for imbedding, 81, 82, 83; for preserving, 5
Xylol balsam, 235, 245

Y.

YAMAGIWA, neuroglia, 442
YOUNG, methylene blue, 206
YVON, test for water, 62

Z.

ZACHARIAS, acetic alcohol, 55; iron-carmine, 146; Flagellata, 481; Copepoda; 454
ZALESKI, iron, 320
ZANDER, test for Chitin, 456
ZAWARSIN, cornea, 336
ZENKER, fixing mixture, 50
ZERNECKE, *Ligula*, 465
ZETTNOW, flagella, 483
ZIEGLER, decalcification, 282
ZIEGWALLNER, glycogen, 320
ZIEHEN, gold and sublimate method, 434
ZIEHL, carbolic fuchsin, 181
ZIELINA, blood, 359
ZIMMER, picro-acetic acid, 58
ZIMMERMANN, A., sieve-dishes, 4; micro-chemistry, 321; nucleoli, 324
ZIMMERMANN, K. W., Golgi's impregnation, 431; bone, 355; connective tissue, 346
Zinc chloride, for fixing, 53; for hardening, 381; for impregnation, 436
Zinc, detection of, 320
Zoantharia, 472
ZOGRAF, Rotatoria, 462; Protozoa, 478
ZOJA, methylen blue, 202; ova of *Ascaris*, 315; Protozoa, 478; bioblasts, 328
ZSCHIESCHE, larvæ of *Alcyonidium*, 449
ZSCHOKKE, benzo-purpurin, 191; cartilage, 357
ZUR STRASSEN, ova of *Ascaris*, 315; *Bradynema*, 463
ZÜRN, retina, 444
ZWAARDEMAKER, safranin, 177

ADLARD AND SON, IMPR., LONDON AND DORKING.